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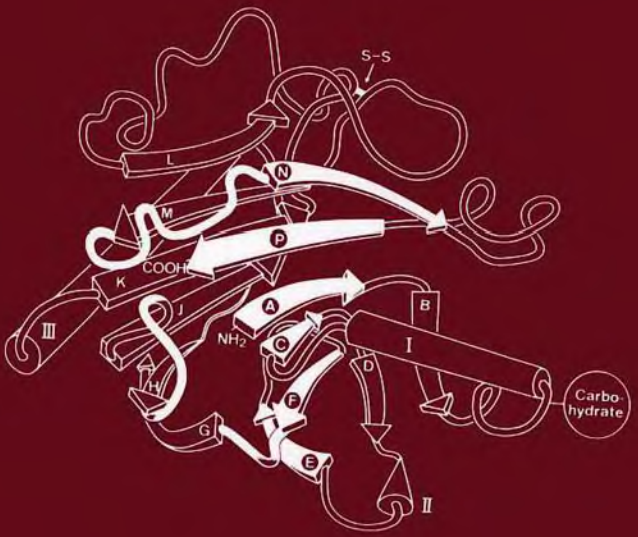
DRUGS AND THE PHARMACEUTICAL SCIENCES VOLUME 84

Pharmaceutical Enzymes



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Pharmaceutical Enzymes

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Preface

Almost two decades have passed since the second edition of *Pharmaceutical Enzymes* was published in 1978. In fact, the need for such a volume never waned; the book was out of print a few years after its initial publication. Since that time, there has not been any monograph or book covering the field. Thanks to continuing demand by many colleagues in industry and academia, we decided to compile an entirely new edition. The scope of pharmaceutical enzymology has expanded over a broad spectrum of scientific fields, and this book is a reflection of these advances. Of paramount importance was the need to tackle the subject in an interdisciplinary fashion so as to appeal to researchers active in drug companies, universities, and pharmaceutical regulatory affairs as well as graduate students and professionals in pharmaceutical sciences and medicine.

Molecular and biochemical data concerning pharmaceutical enzymes are presented together with robust methods of measurement and evaluation, and when relevant information is known, emphasis is placed on pharmacology and clinical impact. Special attention is given to practical, often previously unpublished background information and material not readily found in standard textbooks and other reference works.

This book consists of 15 chapters and is divided into four major sections. Part One addresses the actual knowledge of bioavailability of pharmaceutical enzymes. Part Two presents the spectrum of different chemical approaches for tailoring enzymes to improve therapeutic effectiveness. It features a stepwise and practical guide to molecular modeling of important pharmaceutical targets, including flowcharts, examples, and references to further sources of information in databases and the literature.

Part Three reviews representative multienzyme compositions, such as pancreatin, as well as promising recent developments with glucocerebrosidase, deoxyribonuclease and protein inhibitors of elastase. This part integrates biochemical, experimental, and clinical data of therapeutic enzymes, such as asparaginase, bromelain, hyaluronidase, and cysteine proteinases, about which

pharmaceutical information in a pharmaceutical context is rarely available in the literature.

The last part brings together a completely updated and elaborate description of the methods of assay of pharmaceutical enzymes, reflecting the output of sustained collaborative work between universities and industries in the framework of the Commission on Pharmaceutical Enzymes of the Fédération International Pharmaceutique during the last two decades.

As editors, we owe thanks to many for advice, encouragement, and help: first to the contributors, for sharing their expertise; to many colleagues around the world, for their willing cooperation and extensive collaborative work in the development of the methodological aspects of this volume; to the former and present members of the International Commission on Pharmaceutical Enzymes, for their continuous support during more than three decades; to Dr. M. van Sande, for being a constant source of support throughout the writing of the book; to Ms. L. Pelosi, M. Snoeckx and K. Wullsert for excellent assistance during the production of this book.

Albert Lauwers
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1

Biopharmaceutical Aspects of Enzyme Absorption

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I. INTRODUCTION

Enzymes as therapeutic products are not only very diverse but also very complex. They are proteins, a large group of pharmacologically active molecules that also includes monoclonal antibodies, cytokines, growth factors, hemostatic and thrombostatic agents, and vaccines.

Problems in the therapeutic application of proteins are caused by host contaminants, species specificity, cross-reactivity, immunogenicity, and the difficulty of targeting the right location in the human body. Moreover, the rate at which proteins reach the plasma compartment depends upon the route of administration: intravenous or subcutaneous injection and sometimes pulmonary delivery.

The gastrointestinal tract is the most appropriate route for drug delivery. However, for enzymes, other proteins, and peptides, this way of administration is a new challenge for many research groups. Indeed, the parenteral route is often the preferred one for protein and peptide administration. Despite current opinion that the gut is completely impermeable to proteins and peptides, progress has recently been made in the elucidation of possible routes of penetration through the intestinal barriers. There is now evidence that small quantities of peptides and proteins can be absorbed intact from the gut.

This chapter will discuss some biopharmaceutical aspects of enzyme absorption, although most studies have been performed using other proteins or peptides. Small molecules are predominantly metabolized in the liver, but proteins and peptides are degraded by enzymes present in most compartments of the body.

II. GASTROINTESTINAL PATHWAYS FOR DRUGS

A. Oral Cavity

The oral cavity has little importance in the liberation of most drugs. Mastication mainly causes mechanical destruction and size reduction. A salivary volume of about 0.5–1.5 L, a pH of 6, and a surface tension of 15–20 mN/m are important factors for drug delivery; the membrane surface pH is 6.7. No peptide bond–splitting enzymes are present.

In optimizing peptide or protein delivery, buccal administration is sometimes preferred. The buccal membrane is a stratified squamous epithelium, and the intercellular spaces are filled by cellular extrusion products.

B. Esophagus

The esophagus has a pH of 5–6, and its absorptive capacity is negligible.

C. Stomach

Epithelial cells secrete hydrogen carbonate ions, which have a cytoprotective action. They neutralize the hydrochloric acid secreted by the parietal cells (about 0.87 M) in the mucus layer, creating a neutral to slightly alkaline pH at the stomach wall. About 2–3 L of fluid are produced per day with a pH of about 0.8–2.0. After food intake, the pH rises to about 4–5, but further gastric secretion restores a strongly acid pH within 30–45 minutes [1]. In about 40% of patients above 65 years old, achlorhydria is observed, which increases the pH. In neonati, the pH is neutral to slightly alkaline. The acidic conditions denature protein molecules, exposing them to hydrolysis by endogenous pepsin, which is an endopeptidase unable to cause complete proteolysis. It is an aspartic proteinase that is most active at pH 2–3 and inactivated at pH above 5. Very large polypeptides with molecular weights of several thousand are mainly formed that are not absorbed in the stomach. This inactivation can be reduced by administration of antacids, such as sodium hydrogen carbonate, or by H_2-receptor antagonists such as Cimetidine®. The administration of pepsin-containing drugs improves food digestion. Other enzyme preparations are often administrated using coatings that resist stomach fluid.

D. Stomach Emptying

How long drugs stay in the stomach can influence their bioavailability. Rapid stomach transit can be achieved by administrating the drug in a large volume of liquid that is warm, isotonic, and slightly alkaline and that is taken ambulatory on

an empty stomach. The liquid can be taken before and up to 1 hour after drug administration.

E. Gut

1. General Pathways for Drug Absorption

The structures of the gut are differentiated and optimized for the primary biological functions—the digestion and absorption of food. They are a compromise between two contradictory requirements: the availability of a large absorptive surface and a small abdominal volume. Enzymes can influence the morphology of the intestinal tract. Amylase-supplemented diet significantly increases the villus length within the jejunum and ileum [2]. The pathways followed in general by drug molecules during absorption are shown in Figure 1. All steps during the absorption process are potential barriers with the ability to decrease the biological availability of the drug, especially when labile molecules such as enzymes are involved. We will review them in chronological order, the heading letters being keyed to Figure 1.

a. Drug molecules in solution

When entering the duodenum, drug molecules are often already in the dissolved state.

b. Gut lumen

Enzymes can be liberated from enteric-coated formulations by the neutral to slightly alkaline pH caused by secretions from the pancreas, the gall bladder, and the intestinal mucosa. They have a hydrogen carbonate concentration of about 85 mM, which causes an increase in pH. Bile salts decrease the interfacial tension, promoting the liberation of drugs from solid dosage forms.

After meals containing proteins, free amino acids are found in the intestinal lumen. They do not originate entirely from the digestion of proteins, as some amino acids generated by brush border hydrolysis can diffuse back to the intestinal lumen.

Digestive enzymes are important barriers to peptide and protein absorption [3]. They are able to digest protein drugs and include trypsin, α-chymotrypsin, elastase, and carboxypeptidase A. The first three enzymes are able to cleave internal peptide bonds in many proteins: (1) trypsin has more affinity for bonds near basic amino acids, such as arginine and lysine; (2) α-chymotrypsin cleaves peptide linkages near hydrophobic amino acids, such as leucine, methionine, phenylalanine, tryptophan, and tyrosine; (3) elastase cleaves near alanine, glycine, isoleucine, leucine, serine, and valine. Shortly after eating, about 200–800 μg of trypsin and α-chymotrypsin are present in the human duodenum.

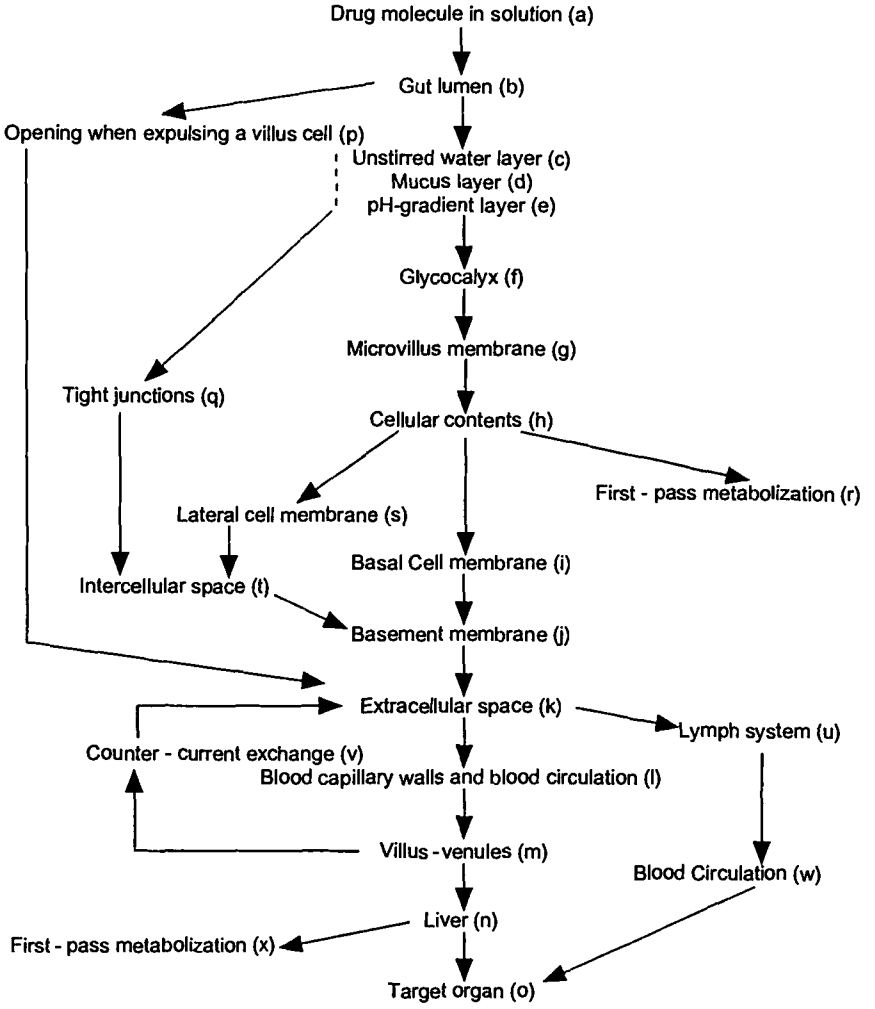

Figure 1 General pathways for drug absorption in the gut.

c. *Unstirred water layer*

Adjacent to the mucosa, no agitation is possible and the transport rate of most drugs is determined by passive diffusion in the aqueous medium. The thickness of the layer in situ amounts to about 500 μm and in vitro to about 150–200 μm. The average unstirred layer thickness can be calculated from Michaelis-Menten constant and maximal velocity of maltose hydrolysis by intestinal maltase. In

celiac patients it increases from about 25 μm to about 170 μm [4]. The diffusion coefficient of drugs with molecular weights above 250 through this layer decreases proportionally with the cube root of the molecular weight due to Brownian motion according to the Einstein's law. When there is enough intestinal motility, the thickness of this layer decreases to the thickness of the mucus layer.

d. Mucus layer

A layer of mucus covers the gastrointestinal tract and protects the underlying cell membrane from the corrosive elements in the luminal content. It is a part of the unstirred water layer. This mucus is composed mainly of water; glycoprotein molecules constitute about 3% of native mucus. The thickness is between about 50 and 450 μm, and it is a very important barrier to drug absorption [5]. Mucus forms a viscoelastic gel and is completely elastic when stress is applied for a short time. It recovers completely after deformation. At higher stresses, e.g., when solid particles pass, it has the capability of viscous flow. Glycoproteins have a protein core with oligosaccharide side chains attached to specific amino acid residues. They have molecular weights of several hundred thousand, and the core proteins can be subjected to proteolytic action of endogenous proteases or protease-containing drugs. Binding of drugs to mucin decreases their absorption rates, which has been the subject of many research papers. Due to hydrogen bonding to mucin, ergot peptide alkaloids have incomplete absorption [6]; tripeptides bind to mucus, but the negatively charged molecule benzoic acid does not [7]; solutes with molecular weight > 4000 have a significantly lower diffusion coefficient in mucus than in water [8]. Many questions concerning the influence of the mucus layer on drug diffusion remain unanswered.

The thickness of the mucus layer changes in function of age. In rats a subsequent increase of microclimate pH from 6 to 6.5 is observed with increasing age [9].

The influence of gastrointestinal mucin on enzyme absorption is not well documented, but it is an intestinal diffusion barrier for many nutrients [10].

e. pH-gradient layer

In contrast to the stomach, where there is a protective pH-gradient from 7 to 1 between the mucosa and the lumen here, there is only a slight pH gradient from about 6 (mucosa) to about 7 (lumen). It corresponds to the inner mucosal part of the unstirred water layer. The thickness of this layer is about 20 μm. The pH has no significant influence on the absorption of protein or peptide drugs.

f. Glycocalyx

Above the absorptive cell membrane (Fig. 2), a layer of mucopolysaccharides form a web of branchings called the glycocalyx. The mucopolysaccharides

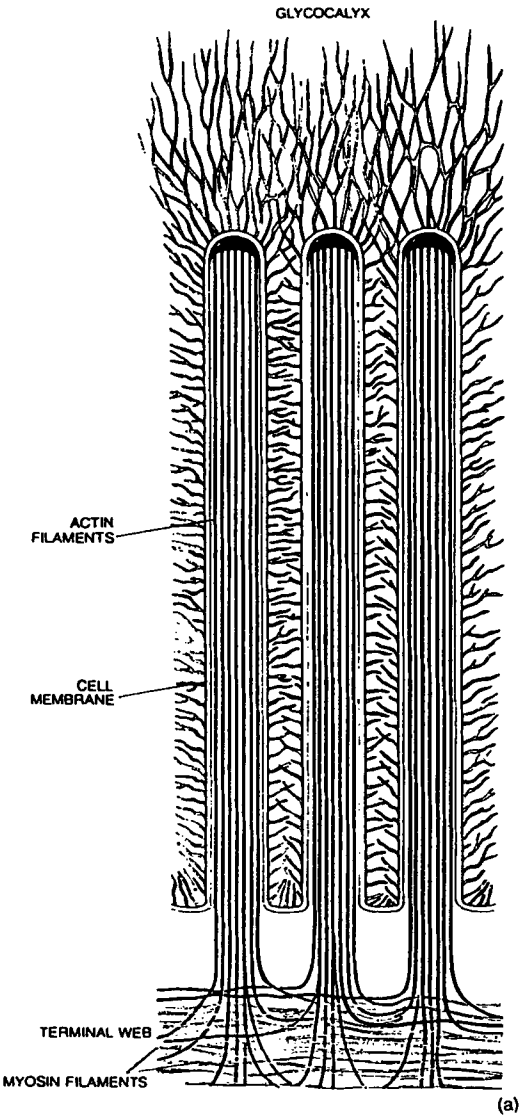

Figure 2 The structure of the absorptive cell of the intestinal lining reflects its specialized function in digestion. The absorptive cell is one of the two main cell types that constitute the epithelium of the villus. The other type is the goblet cell, which secretes mucus. Cells of both types are produced by cell division in the crypts and assume their mature structure during a 2-day migration to the villus tip. Exposed in transit to the action of digestive fluids, some of the membrane covering the absorptive cell may be sloughed off. The cell is abundantly supplied with mitochondria, which provide energy for metabolic

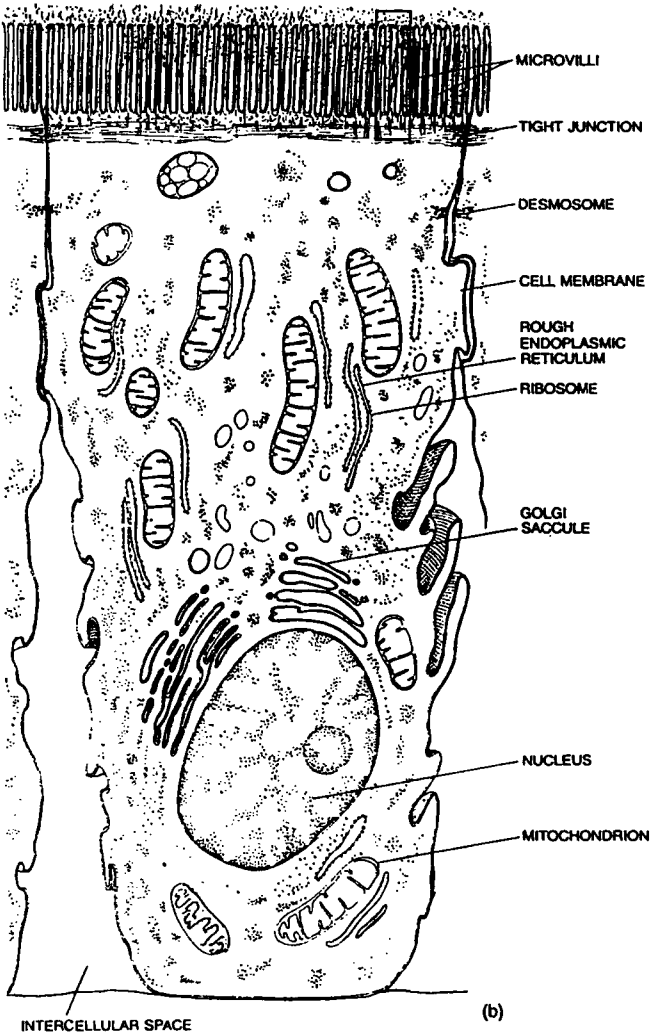

Figure 2 (continued)
processes; with endoplasmic reticulum, where proteins (including digestive enzymes) are assembled; and with Golgi saccules, where carbohydrate side chains are attached to some proteins. Tight junctions and desmosomes bind the absorptive cells into a single sheet as they traverse the villus. The nucleus lies deep in the cell. The most striking feature of the cell is the brush border, a prominent structure on the luminal surface of the cell, which is made up of rows of minute projections called microvilli. In the core of the microvillus is a bundle of fibers composed of protein. The fibers from all the microvilli on each cell extend down to the terminal web, a meshwork of fibers running parallel to the luminal surface of the cell. (From Ref. 7.)

are side chains of glycoproteins localized at the absorptive cell microvillus membrane. The protein part of the glycoprotein penetrates to varying depths into the lipid double layer of the membrane, depending on the protein. Many proteins have a transport function and/or enzymatic function for food-digestion products. Absorption of drug molecules, however, is only slightly mediated by these protein-carrier molecules. Aminopeptidases, which are able to remove an amino acid from one end of a short peptide chain, are embedded in the membrane and pass all the way through the membrane to the interior of the microvillus [11].

g. Microvillus membrane

The microvillus membrane of the absorptive cell has a thickness of 9.5–11.5 nm, which is somewhat thicker than normal biological membranes. It is folded into "microvilli" (about 200,000/mm^2 in the human jejunum [11]), and increases the surface of the intestine by a factor of 20. Microvilli contract, and, during contraction the fibers in the core slide down toward the underlying terminal web. In this region, the movement of drugs and other molecules will not only be diffusive but also agitative. Molecules passing the membrane are absorbed by the "transcellular" pathway.

h. Cellular contents

In humans, it is very difficult to show morphologically transcellular transport of intact proteins. Walker's group [12] has done many studies on intestinal uptake of macromolecules and has provided direct morphological and physiological evidence for macromolecular transport using horseradish peroxidase as the transport marker. This enzyme is a glycoprotein with a molecular weight of about 40,000 and can be visualized by electron microscopy or detected by ELISA techniques. It has been found within membrane-bound cytoplasmic canalicular, vesicular, and vacuolar structures. When proteins and peptides are taken up by cells, they must exceed the lysosomal digestive capacity, i.e., the first-pass intracellular metabolization, to be transported out of the cell.

i. Basal cell membrane

The basal cell membrane has a barrier function similar to that of other biological membranes. After passing this membrane, drug molecules enter the subcellular space.

j. Basement membrane

The basement membrane has a thickness of about 30 nm and is composed of mucoproteins. The permeability for proteins and peptides is very good, and even lymphocytes and chylomicrons pass without restriction.

k. Extracellular space

The extracellular space is in very good contact with a very dense network of blood capillaries.

l. Capillary walls and blood circulation

Most drug molecules are transported to the target organ by the circulating blood, but there are serious barriers to protein transport both entering and leaving the blood circulation. The barrier function of the endothelium monolayer is discussed elsewhere in this book [13]. The gastrointestinal tract accounts for about a third of the total blood circulation. Moreover, the total capillary length of the human body is estimated to be about 95,000 km. This very differentiated network can be estimated from the fact that, on average, five mucosal cells are present between two capillary connections. In most cases, the intestinal circulation is a virtual sink for drug molecules. In the blood, enzyme inhibitors are present. Enzymes that should have their therapeutic action in the blood (e.g., clot lysis) can be hindered by inhibitors, while the bioavailability of others is increased by inhibition of protease activity. Studies of the distribution of macromolecules in the blood are particularly difficult to perform in humans, although many uncertainties could be solved by such research. The presence of active proteases and peptidases with broad specificity is often neglected, and the degradation of protein in circulation has certainly led to an underestimation of the absorptive capacity of the intestinal tract for intact protein [14].

m. Villus venules

Most absorption takes place at the top of the villi, and drug concentration is much lower in the crypts between the villi. Therefore, when drug molecules are absorbed at the top and are transported to the lower parts of the villus—the venules—they can exit the venules where the drug concentration is low.

n. Liver

Before reaching the target organ, drugs must pass through the liver. This can be avoided by direct intravenous delivery of drugs. Optimized oral or rectal delivery also avoids the liver passage.

o. Target organ

In the target organ the intact protein or drug molecules again have to cross biological barriers.

p. Opening when expelling a villus cell

Epithelial cells migrate from the crypts to the tips of the villi in about 2 days [11]. During this time they differentiate and, at the end of their life cycle, are shed into the lumen. Their contents are extruded in the succus entericus and the intracellular enzymes become available for food and drug metabolization in the gut. The amount of cellular material liberated is about 250 g per day. Drugs and macromolecules can be absorbed by this first "paracellular" pathway, but no quantitative data are as yet available concerning the extent of this phenomenon. Recent literature suggests that some authors are in favor of absorption between the cells, called persorption and responsible for the uptake of cells [15], while others are still skeptical.

q. Tight junctions

When drug molecules can pass the tight junctions between the cells, they can be absorbed via a second "paracellular" pathway. There is some controversy about the permeability of the tight junction. Moog [11] argues that they are not permeable at all, as the jejunum and the ileum have a high ionic conductance and the transepithelial flow is mainly paracellular [16]. The equivalent pore diameter of the tight junctions has been estimated by means of triaminopyrimidine to be 1.2 nm [17]. Osmotic studies indicate that the pores differ in size and that cation-selective pores exist in parallel with neutral pores with an equivalent diameter of 13 nm [18]. Both in vitro and in vivo glucose reversibly increase the permeability of the intestine, probably through a perijunctional "muscle" ring opening in the tight junctions [19]. Pharmacologically significant amounts of peptide absorption may be achieved in this way [20]. In many diseases paracellular flow increases in humans [21].

r. First-pass metabolism by the cellular contents

The same enzymes that are found in the liver are present in small amounts in mucosal cells. They catalyze oxidations, reductions, hydrolysis, and conjugations. Specific activities of the enzymes are lower than in the liver, but the activity can increase by induction. For many drug molecules, the concentration is higher than in the liver, so there is a higher number of reaction cycles per time unit and per mole of enzyme. Proline endopeptidases cleave the Pro-NH_2 bond in proteins and peptides [22].

s. Lateral cell membrane

The lateral cell membrane has a barrier function similar to that of the basal cell membrane. After passing this membrane, drug molecules enter the subcellular space.

t. Intercellular space

This space has a very good permeability for larger molecules such as proteins.

u. Lymph system

In each villus, a central lacteal is found and more lipophilic drugs can be absorbed via this route. The target organ is reached through the ductus thoracicus and, therefore, without passing through the liver, thus avoiding hepatic first-pass metabolism (n, Fig. 1).

v. Countercurrent exchange

As indicated in Figure 1 (m), drug molecules can exit the villus venules. The global effect of this exchange is a decreased absorption capacity of the blood circulation. When the concentration of absorbed material is high, as is the case in food absorption, the efficiency of the blood supply is decreased by a factor of up to five. The effect on protein and peptide absorption has not been studied but is probably negligible.

w. Blood circulation

Protease inhibitors will decrease the possible degradation of proteins.

x. First-pass metabolization

As in the intestinal cells (r, Fig. 1), the presence of digestive enzymes in the liver causes metabolization of many drugs, including proteins and peptides.

2. Absorption by Membranous Epithelial Cells

a. Introduction

Section 1 describes the standard absorptive pathways for the absorption of food, most drugs, and to a minor extent, proteins and peptides. Membranous epithelial cells are much less numerous than absorptive cells, but they are of special interest since they have structural properties that could be favorable for protein absorption.

The standard absorptive pathways for the absorption of food, most drugs, and, to a minor extent, proteins and peptides have been described above. Membranous epithelial cells are much less numerous than absorptive cells, but they are of special interest since they have structural properties that could be favorable for protein absorption.

b. The membranous epithelial cell

Membranous epithelial cells (Fig. 3), M-cells or microfold cells, are localized in regions overlying lymphoid follicles, which are known as Peyer's patches

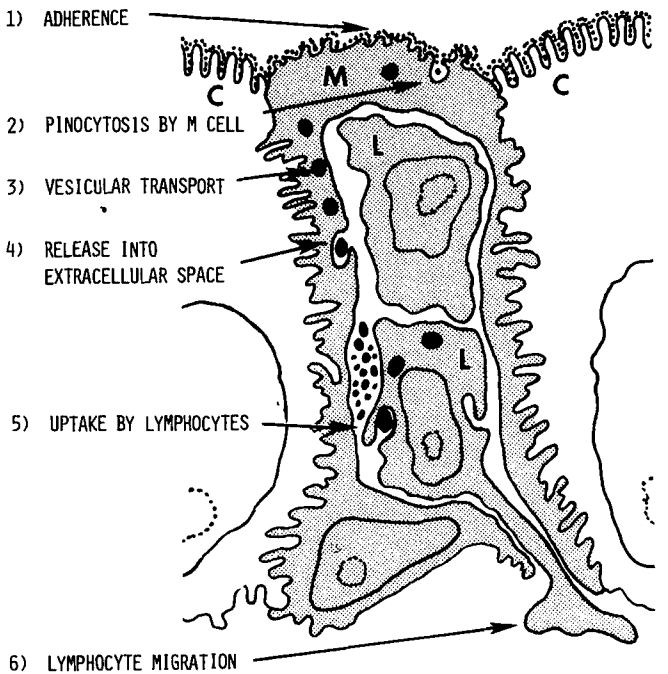

Figure 3 Diagram summarizing the stages observed in the transport of horseradish peroxidase (HRP) by the M cell from the intestinal lumen to the intraepithelial lymphocyte. C: Columnar cells; L: lymphocytes. (From Ref. 24.)

[23]. Their main function is the sampling and presentation of antigens to the luminal tissue. In comparison with absorptive cells, they have a diminished glycocalyx. They also have short, irregular microfolds at the apical surface, few microvilli, many vesicles in the apical cytoplasm, and few lysosomes [24]. Antigens can be present in intestinal lymphoid tissue by the transport of macromolecules across M-cells. The enzyme marker horseradish peroxidase is transported across M-cells from the lumen to the extracellular space [25]. The mechanism of horseradish peroxidase is by nonspecific pinocytosis [26]. Very little is known about the metabolization of macromolecules in M-cells. Extracellular proteins are first trapped in fluid droplets in the invaginations of the noncoated regions of the cell, which are then internalized [27].

III. BIOPHARMACEUTICAL TECHNIQUES TO ENHANCE PROTEIN OR PEPTIDE ABSORPTION

A. Prevention of Degradation in the Stomach

Degradation in the stomach can be prevented by using enteric-coated galenic preparations. Enteric coating is often used for the delivery of pancreatic enzymes to patients with cystic fibrosis or pancreatic insufficiency. Enzymes are protected from degradation, and the liberation occurs in the gut. When high-strength pancreatic enzyme formulations were used in children with cystic fibrosis, stricture of the ascending colon and histopathological changes of postischemic ulceration repair were found [28]. Intact enzyme microspheres of pancreatic enzymes can come into contact with colonic mucosa. However, a cause-and-effect relationship remains to be established [29]. Indeed, a higher dosage of pancreatic enzymes can decrease the discomfort of young patients, some of whom are administered more than 100 low-dose capsules a day.

B. Prevention of Degradation in the Gut

Degradation in the gut is caused by endogenous proteases such as trypsin and α-chymotrypsin. Entrapment of macromolecules in liposomes results in better protection against proteolysis [30]. Since lipid-soluble molecules are absorbed quickly in the gut, it could be expected that liposome delivery will give enhanced absorption by diffusion. However, this problem is not simple, as illustrated by the lack of uptake of liposomes by enterocytes [30,31]. Insulin entrapped in liposomes is absorbed in this way [32].

Pancreatic enzymes strongly effect the stability of proteins and peptides, but absence of pancreatic juice also changes the absorptive properties of the gastrointestinal tract [33].

Several methods have been used to improve the stability against proteolytic enzymes [34]: substituting unnatural amino acids, including D-amino acids, for L-amino acids in the primary structure; introducing conformational constraints; reversing the direction of the peptide backbone and inverting the chirality of each amino acid; or acylating or alkylating the N-terminus or alteration of the carboxy terminus by reduction or amide formation. Peptides are more easily transformed, and whether or not the pharmacological activity is decreased must be determined.

Mucoadhesive polyacrylates are promising excipients to inhibit the proteolytic activity of intestinal enzymes with an enhanced stability of peptide drugs as a result [35]. But, the influence on the absorption of proteins and enzymes remains to be seen.

Coadministration of protease inhibitors can optimize protein absorption. The administration of soybean trypsin inhibitor improves the absorption of insulin

from the ileum of the rat [36]. With aprotinin both insulin and RNAase were recovered from the ileum, respectively, 30 and 5 times better than control values [29]. Other useful protease inhibitors include bacitracin, amastatin, bestatin, puromycin, and a series of derivatives of α-aminoboronic acid [38]. The boron atom can form a boronate ion that is believed to resemble the transition state of aminopeptidase substrates.

It is probable that proteases such as trypsin and bromelain, which are somewhat stable to self-proteolysis, would be absorbed to a greater extent than other proteins of comparable size. Coadministration of soybean trypsin inhibitors or pancreatic duct ligation increases the absorption of intact protein [39].

C. Enhancement of Mucosal Penetration

1. Penetration of the Unstirred Water Layer and the Mucus Layer

As noted in Section II.E, both layers decrease the absorption rate, especially of large water-soluble molecules, since the diffusion coefficient is directly proportional to the reciprocal values of both the hydrodynamic radius and the viscosity of the layers. About the influence of absorption enhancers on mucus rheology very little is known. Bile salts and phospholipids are known to reduce the viscoelasticity of bronchial mucus and probably also of mucus layers in the gastrointestinal tract [40]. The results should be interpreted with caution since it is not the observed (macro)viscosity that determines the diffusion rate in polymer networks but the much lower (micro)viscosity between the polymer chains [41].

Polyacrylic acid polymers can inhibit luminal degradation of insulin and other large peptides by trypsin and chymotrypsin. They have strong bioadhesive properties, increasing the in situ absorption of insulin [42].

Absorption enhancers have only restricted efficacy in increasing peptide or protein absorption but often alterate the cellular morphology of the gastrointestinal tract [43].

2. Transcellular Transport Enhancement

By altering the nonprotein thiol levels in the membranes, penetration enhancers like salycilate might promote the uptake of hydrophylic compounds [44]. Most of the studies, however, have been concerned with nonprotein or nonpeptide drugs.

3. Paracellular Transport Enhancement

The main advantage of paracellular transport is the low proteolytic activity present in the paracellular pathway. Lipid-surfactant mixed micelles enhance the

permeation of dextran [45]. Both the expansion of the tight junction—e.g., by lowering extracellular calcium ion concentration—and of the intercellular space and the increased water influx through that space are observed [38]. Penetration enhancers can have an undesirable and acute damaging effect on mucosal membranes and also increase the permeation of environmental toxins.

IV. UPTAKE OF PROTEINS BY THE NEWBORN

Most cells have some capacity to endocytose macromolecules. The acquisition of immunity by absorption of maternal IgG molecules before and immediately after birth is very important. Before gut closure, the endocytotic capacity is very large, and even adults retain a small amount of endocytotic capacity. The macromolecules are first encapsulated and then internalized in vesicular bodies, endosomes, or phagosomes. The mechanisms of macromolecular absorption in the proximal and distal intestine are given in Figure 4 [24]. These bodies move into the inside of the cell, where they fuse with lysosomes in which digestion can occur. A small percentage of macromolecules escape digestion [44]. Milk proteins, bovine serum albumin, and β-lactoglobulin penetrate the mucosal barrier [46]. The uptake of γ-globulins is selective, while other macromolecules are transported in smaller quantities by a nonselective pinocytotic mechanism [12].

V. UPTAKE OF PROTEINS IN PATHOLOGICAL SITUATIONS

Increased uptake of large molecules can be expected in all pathologies where the gut is damaged or where the structure of the villi is changed. In patients with eczema and food allergy, the intestinal permeability of the probe molecule polyethylene glycol is increased [47]. In infants with α_1-antitrypsin deficiency, trypsin can be transported to the systemic circulation and reach the liver, causing inflammation and eventually fibrosis [48]. An increase in pinocytosis is also present in malnutrition.

VI. EXPERIMENTAL EVIDENCE FOR THE UPTAKE OF ENZYMES AND PROTEINS

The study of the oral bioavailability of enzymes is very complicated. When using radiochemical methods, the amounts of absorbed enzymes are overestimated,

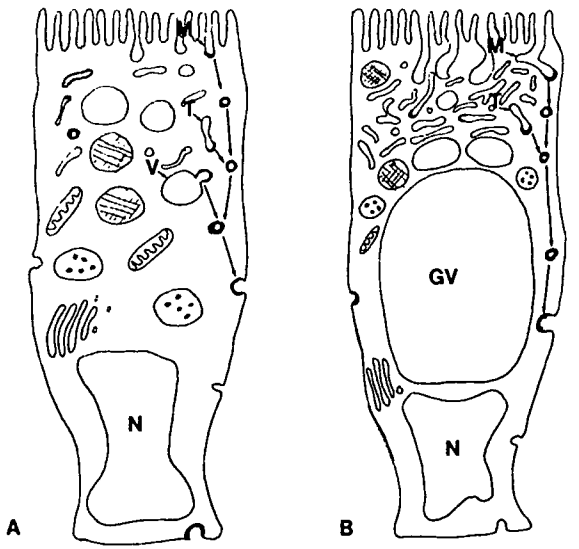

Figure 4 Transcellular transport in the proximal and distal intestine of the suckling rat. (A) In the proximal intestine, macromolecules may be selectively absorbed in coated pits along luminal membranes (M) and transported via coated vesicles to the basolateral surface (arrows). Alternatively, selection may occur in tubular compartments (T) or from endosomal vesicles (V). (B) In the distal intestine selective absorption may occur in coated pits at the luminal membrane (M) or from the numerous tubules (T) of the elaborate endocytic complex. (From Ref. 18.)

while immunological or enzymatical methods of absorption are underestimated. Serum-proteinase inhibitors in particular negatively influence the activity of absorbed proteases.

Proteolytically active bromelain is found in human volunteers after oral intake and is partially bound to certain plasma proteins, two of which are identified as α_2-macroglobulin and α_1-antitrypsin [49]. The techniques used in this study are based on both biological and immunological methods.

The flux of horseradish peroxidase from the mucosal to serosal sides is investigated by in vitro experiments in stripped goldfish intestinal epithelium. Through enzymology and electron-microscopic techniques the intestinal uptake is shown [50].

Compounds with a molecular weight up to 4000 might be significantly absorbed [51]. Papain, bromelain, trypsin, and chymotrypsin significantly enhance the transport of macromolecular markers, independent of their molecular

weight across Caco-2 cell monolayers. The markers are FITC-dextrans with molecular weights of 4 to 580 kDa. Self-enhanced paracellular transport may be the mechanism of absorption [52]. The presence of other proteins also influences the absorption of pharmaceutical enzymes. Caco-2 cells do not produce an unstirred mucin layer and further experiments with mucin producing cell layers should be performed to understand better the in vivo absorption of enzymes.

VII. CONCLUSIONS

The oral delivery route has, despite many problems encountered with bioavailability, attractive properties that deserve to be explored more carefully in the future. Indeed the use of noninvasive delivery routes is recommended by many pharmacologists. Administration of enzyme inhibitors, protective polymers and permeation enhancers, molecular modifications, or entrapment in new delivery devices are techniques now under investigation. Also, delivery through noninvasive routes other than oral administration such as buccal delivery [38] and ocular, nasal, pulmonary, and transdermal delivery [39] are promising, despite their rather low biological availability. It can be concluded that we might expect a better understanding of the biopharmacies of enzyme absorption in the near future.

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2

The Endothelium Monolayer: Barrier to Enzyme and Drug Delivery

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I. INTRODUCTION

In order to function properly, cells in the human body must continuously exchange nutrients and energy with the external environment. The main source of nutrients is the blood stream. To reach the cells, nutrients, growth factors, and hormones must cross the endothelial cell barrier. The complex transport mechanisms taking place at the blood-vessel wall interface are not fully understood. To understand the normal functions and pathological dysfunctions of the endothelium, cellular and molecular mechanisms of endothelial transport of macromolecules must be studied in greater detail. How macromolecules or drug-carrier complexes leave the vascular space is also of great practical significance. Recent data have demonstrated that the biochemical properties of both endothelial cell surfaces and permeant molecules play a significant role in transendothelial transport. Interacting with the endothelial barrier, the macromolecules can be taken up by the cell for their own needs by endocytosis, transported across the endothelial cell to the interstitial fluid by transcytosis, or can leave the circulation through intercellular junctions by paracellular transport. These different mechanisms may be involved in organ-specific drug delivery in which access to the target tissue may use distinct chemically mediated mechanisms for transendothelial transport. In this chapter we summarize the main characteristics

of the endothelial cells that should be considered when planning specific drug delivery.

II. ENDOTHELIAL CELLS–A SELECTIVE BARRIER

The vascular endothelium is a highly specialized, polarized squamous epithelium (Fig. 1). As a tissue involved mainly in the modulation and control of the transendothelial exchange, the endothelium possesses characteristic structures which are involved to various degrees in the transport of macromolecules. The most prominent feature of endothelial cells is a large population of "plasmalemmal vesicles," which are small membrane-bound vesicles with the mean outer diameter of ~70 nm. They are apparently either free in the cytoplasm or they open to both luminal or abluminal cell surfaces through a neck 10–40 nm in diameter which sometimes seems to be closed by a thin diaphragm displaying a central knob [3,9]. Biochemical composition data allowed Simionescu et al. [83] to postulate that plasmalemmal vesicles, although morphologically identical, contain at least two functionally distinct entities: pinocytic vesicles, which perform "endocytosis," and transcytotic vesicles, which transport the probes across the cell by "transcytosis." When one vesicle or a chain of vesicles opens simultaneously on both surfaces of the cell, a "transendothelial channel" is formed. These structures are presumed to behave like dynamic hydrophillic pathways that can function as either small or large pores [84]. Besides vesicles and channels, the

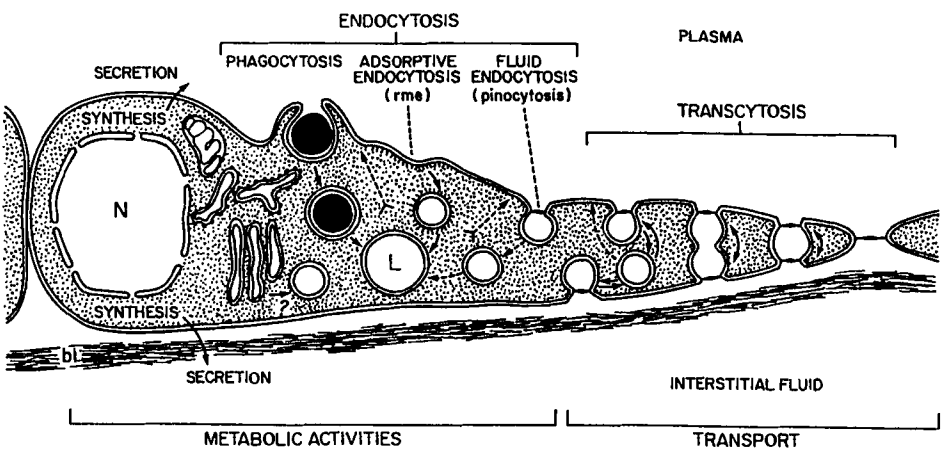

Figure 1 Vascular endothelium.

visceral capillaries possess other unique features, the "fenestrae." These are round openings with a diameter of ~70 nm that have thin diaphragms (as in intestinal mucosa and pancreas capillaries) or are diaphragm-free (as in renal glomerulus and adrenal cortex capillaries) [85,86]. The fenestral diaphragms are polarized cellular components that contain strong anionic residues [5,88] and receptors for wheat germ agglutinin [89] on their luminal front. Clathrin-coated pits and coated vesicles are structures present in both continuous and fenestrated capillaries, and together with endosomes, multivesicular bodies and lysosomes are the structural components of the endocytotic pathways in the endothelial cell. On the other hand, the highly dynamic structures like plasmalemmal vesicles, transendothelial channels, and fenestrae are particularly involved in the transport processes [9]. Under the influence of local physiological conditions, the cells have undergone specific segmental differentiations expressed as a large phenotypic heterogeneity with characteristic features for the endothelium of large vessels versus that of microvessels. Significant differences are also present between the arteriolar capillary and venular endothelium. The structural differentiation is mainly expressed by a significant attenuation of the cells, an unusually high volume density of plasmalemmal vesicles, and relatively simplified junctional complexes [1,2].

It is well established that the major contribution in the micro- and macromolecule exchange between blood plasma and interstitial fluid belongs to the microvascular beds. According to the characteristic features associated with the specific function of the host tissue or organ, capillary endothelia have been classified as continuous or nonfenestrated (in heart and the majority of blood vessels), fenestrated (in visceral organs), and discontinuous (in hematopoietic tissues and liver) [1,3]. The discontinuous capillaries have thin walls, and the endothelium and basal lamina have large gaps up to thousands of Angstroms in size allowing water and small molecules to pass freely to the interstitial space. In fenestrated capillaries, free transport is restricted by the fenestrae. These nonhydrophobic barriers are responsible for the transport of water and small solutes across the endothelial barrier. In the continuous endothelium found in the majority of blood vessels, the cells form a highly selective barrier to macromolecules due to the occluding junctions between the cells, also subtended by a continuous, well-organized basement membrane. Significant modulations also exist within the same type of capillary endothelium such as brain capillaries, which have rare plasmalemmal vesicles and very tight endothelial junctions, or myocardial capillaries, which have a high density of plasmalemmal vesicles and special tight intercellular junctions [4]. Moreover, heterogeneity can be detected within the same endothelial cell, e.g., the presence of strong anionic sites on coated pits and fenestral diaphragms and their low occurrence or absence on plasmalemmal vesicles and their diaphragms [5]. The anionic sites of fenestral diaphragms on the lumal front are primarily represented by heparan sulfate,

whereas the abluminal aspect has a neutral charge [87]. Also, the plasmalemmal vesicles, transendothelial channels, and their diaphragms are particularly rich in β-D-galactose and β-*N*-acetylglucosamine [6] (Fig. 2). The preferential distribution of some proteoglycans and monosaccharide moieties define on the endothelial luminal front biochemically distinct microdomains, which are maintained by protein interactions either in the membrane itself or between the membrane and stabilizing cytoskeleton elements located on the cytoplasmic side of the plasmalemma [7,8]. The intercellular junctions of vascular endothelium are less elaborate than those of other epithelia. Desmosomes are generally missing, and the two types of junctions present are the occluding (tight) junctions and communicating (gap) junctions. Segmental variations of the junctions have been identified; among them the most striking is found at the level of the postcapillary venular endothelium. These junctions are poorly organized, partially open, and permeable by electron opaque tracers [10,11]. Communicating junctions are not found in capillary venular endothelia but are present in the endothelium of muscular venules, veins, arterioles, and arteries [10]. In arterioles and arteries the junctions are well organized and intercalated among the occluding zonule. The capillary endothelium displays occluding junctions only. The physical forces and local physiological needs of the tissue dictate the organization and complexity of the intercellular junctions. As a general rule, within the microvascular endothelium the intracellular communication is well developed in arterioles as compared with venules and capillaries, and the intercellular sealing is strong in arterioles, moderate in capillaries, and very loose in venules.

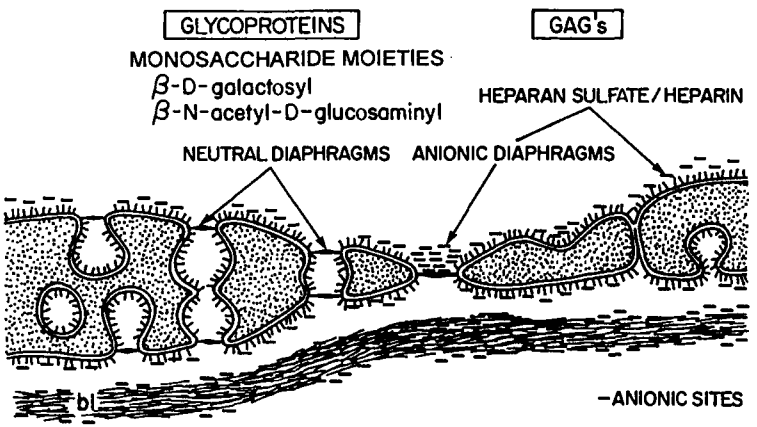

Figure 2 Endothelial cell surface biochemistry: microdomains of glycoconjugates. GAGs: Glycosaminoglycans.

III. ENDOTHELIAL TRANSPORT

The majority of substance exchange takes place in microvascular endothelium where the vessel wall is reduced to the endothelial cells and the basal lamina. Molecules, particles, and cells can cross the monolayer either by paracellular pathways or transcellular by transcytosis (Fig. 1). Like other epithelia, the endothelium plasma membrane is highly permeable to lipid-soluble molecules and gases and by paracellular passage of molecules and ions up to 10 Å in diameter. Particles and cells exit the blood via the venular endothelium. Thus lymphocytes emigrate from the vasculature mainly at specialized postcapillary venules in lymphoid tissues, where the endothelium usually has a characteristic plump morphology, returning to the blood via the lymphatics after several hours [12]. Haselton et al. [13] propose a mechanism by which autacoids (bradykinin, thrombin, adenosine, ATP, and β-isoproterenol) regulate endothelial monolayer permeability. Using endothelial cells cultured on microcarrier beads, the authors showed that the first step in the increase of junctional permeability is the binding of the ligand to specific cell surface receptors. This information is transmitted by second messengers (e.g., diacylglycerol, calcium, or cAMP) to the internal regulatory system of the cell, leading to specific cytoskeletal changes, which affect transmembrane junctional components and finally the junctional permeability. The endothelial monolayer responses are rapid and reversible at physiological concentration of autacoids. Leukocyte emigration is also greatly increased at sites of inflammation where vasoactive amines (e.g., histamine, serotonin, bradykinin) are released in the first steps of the process. Two distinct mechanisms by which polymorphonuclear leukocytes (PMNs) cross the endothelium in response to inflammatory stimuli were proposed by Huang et al. [14]: a rapid PMN-initiated pathway, in which PMNs were stimulated by soluble chemotactic factors to migrate across postcapillary venular endothelium and a slow endothelial cell (EC)–initiated pathway. In the latter, inflammatory stimuli (interleukin I, TNF) induce expression of new EC surface molecules which promote PMN adhesion and diapedesis (transmigration) [15,16]. In atherosclerosis, leukocytes emigrate from the blood to the subendothelial space, where an atherosclerotic lesion is initiated. Atherosclerosis is a chronic process occurring in arteries and involves the adherence and transmigration of circulating monocytes into the arterial intima. There is increasing evidence that vascular endothelial cells play an active role in this process by expressing on their surfaces endothelial leukocyte adhesion molecules [17,18].

Endothelial macromolecule transport is a very complex process, which is governed by at least three sets of factors: plasma driving forces, physicochemical properties of the permeant molecules, and properties and metabolic activities of the endothelial cells [19,20]. By the active participation of the endothelium, the

macromolecules are selected according to their size, shape, charge, and chemistry and delivered inside the cell itself by endocytosis or transported across the cell to the tissues by transcytosis (Fig. 3) [9,21]. During this bidirectional process, the endosomal and lysosomal compartments are bypassed and the molecules are released unmodified on the other side. As already mentioned, the microvascular endothelium has an elaborate transport system represented by plasmalemmal vesicles, channels, and fenestrae. The surface charge and biochemical structure of the plasmalemmal vesicles and channels regulate the transcytosis of plasma anionic proteins, while the fenestrae are responsible for the transport of water and small solutes (Fig. 4) [22]. The transendothelial transport of macromolecules can be nonspecific by nonspecific adsorption to vesicle membrane (e.g., heme and decapeptides, cytochrome *c*) and fluid phase uptake (neutral molecules such as glycogen) or specific adsorption to receptors or specific binding sites localized on the membrane of plasmalemmal vesicles. The latter process is known as receptor-mediated transcytosis [9,21].

IV. ENDOTHELIAL CELL RECEPTORS INVOLVED IN TRANSPORT

In order to cross the endothelial cell barrier, the macromolecules or drug-targeting complex must leave the circulation by either intercellular junctions or a mechanism similar to transcytosis. The permeability of the vessel wall could be

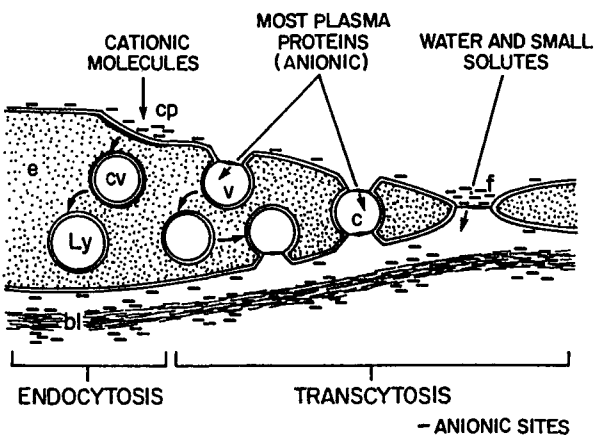

Figure 3 Electrostatic sorting and gating of molecules by the endothelial surface.

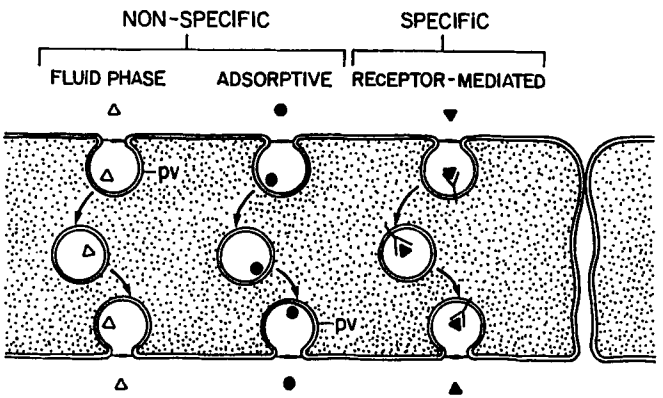

Figure 4 Transcytosis.

regulated by the physiological mechanisms that modulate the transendothelial transport. Endothelial cell monolayers can respond to soluble mediators like histamine [23–25], serotonin [26–28], thrombin [13], and leukotriene C4 [29] by retracting from one another, thereby increasing the permeability of the monolayer to water and macromolecules. These mediators bind to specific receptors on the endothelial cell surface, and increasing the cytosolic free calcium can induce the contraction of cytoskeletal elements and the opening of the intercellular junctions.

PMN binding to and migration across endothelium initiates a sequence of events that resembles that following histamine treatment [14,30], namely, an increase in the permeability of the EC monolayer. For anionic plasma macromolecules, the plasmalemmal vesicles and transendothelial channels are suitable candidates for exit from the vessel lumen. Indeed, receptor-mediated transcytosis of insulin and transferrin has been identified for brain capillaries [31–33]. The insulin carriers have not yet been identified, but it is speculated that they could be coated vesicles and/or plasmalemmal vesicles. Transferrin [34] as well as ceruloplasmin [35,36] binding has been localized to coated pits and vesicles in the endothelium of bone marrow and liver capillary endothelium, respectively.

Hashida et al. [41], using a two-chamber system, proved that cultured porcine aortic endothelia transport low-density lipoprotein (LDL) in a temperature- and energy-dependent process. LDL was also shown to be transported in situ across the aortic and valvular endothelium as well as pulmonary microvasculature [37–39]. In this case transcytosis is a prevalent receptor-independent process by which LDL is transported from plasma to the other cellular components of

vascular tissue to supply their cholesterol needs. The transcytotic process of plasma lipoproteins performed by plasmalemmal vesicles, which shuttles between the luminal and abluminal surface of the cells, maintains cholesterol homeostasis in both plasma and vascular tissue. In hypercholesterolemic animals, the transport and accumulation of both LDL and beta-VLDL by arterial and valvular endothelium is significantly increased, suggesting an increase in endothelium permeability in experimental hypercholesterolemia [38,40,42]. Albumin, the main protein in body fluids, has a biochemical structure able to bind and carry a variety of structurally distinct molecules such as: free fatty acids, thyroid and steroid hormones, bilirubin, bile acids, and drugs [47]. In situ experiments indicated that albumin is taken up and transported across the endothelium by plasmalemmal vesicles [43–46]. It has also been demonstrated that in myocardium [48,49] and adipose tissue [50], the uptake of fatty acids is mediated by an albumin receptor. Experiments performed with albumin-gold conjugates or radioiodinated albumin showed that both microvascular and macrovascular endothelial cells express specific binding sites for albumin represented by albumin-binding proteins (ABP) [51–53]. These proteins exposed on the endothelial cell surface bind specifically and with high affinity to albumin [51,54] and are restrictively located on plasmalemmal vesicles [52]. Recent studies showed that blocking ABP with an anti-idiotypic antibody diminished by 20–25% the transport of 125I-albumin across bovine aortic endothelium in culture, suggesting a receptor-mediated transcytosis of albumin across the endothelial cells. Preliminary results suggested that ABP may selectively discriminate different ligands bound to albumin (fatty acids and hormones) as well as between native and modified albumin [55]. Considering these results, Simionescu et al. [55] suggested a dual role for ABP: as a "docking" protein, which recognizes albumin carrying different molecules, and as a transcytotic receptor, which binds and transports albumin across the endothelial cell.

V. TARGETING EFFORTS

In recent years, there has been a great deal of interest in finding appropriate carriers for delivery of drugs and other biologically active molecules to specific sites of action. The carrier may be a soluble polymer like a monoclonal antibody [56–58], peptide hormone, albumin [65], or sugar derivative [62] to which the drug is covalently bound or a particulate carrier in which the drug is physically trapped as in the cases of liposomes [59,60], albumin microspheres [61,63], or synthetic polymer nanoparticles [64]. The latter has the advantage that the drugs are released by passive diffusion in the desired site of action, while the drugs that are covalently bound to the carrier should be released in an active form by special

mechanisms (enzymatic cleavage). The drug-delivery system must be able to get to the target, but often the target is not accessible because of normal physiological and anatomical barriers. In order to reach a target, the drug should first avoid the reticuloendothelial and immune systems and cross endothelium and basal lamina or the epithelial barriers. The targeted drug must also survive the biochemical modifying systems (e.g., liver acetylation or sulfatation), which may rapidly degrade or inactivate the circulating molecules.

The immune system is highly efficient at clearing foreign molecules or developing an immune response against them. As drugs become more complex, when making them more specific, the potential to be recognized by the host increases proportionately [66]. The particulate carriers are also rapidly removed from the blood circulation by the macrophage cells in the liver, spleen, and bone marrow (mononuclear phagocytic system, or reticuloendothelial system (RES)), thus limiting their prospects for transporting drugs to sites of disease beyond the RES.

The endothelium and basement membrane form a barrier that is difficult for the targeted drug to cross (see Fig. 5). Two plasma membranes and the cell body must be passed before the complex reaches the subendothelial space. Permeation of molecules across the rest of the vessel wall is largely influenced by the biochemistry of the endothelial basal lamina, extracellular matrix, and interstitial fluid. To succeed in the delivery of chemotherapeutic agents to the site of action, the physiological mechanisms of the macromolecule transport should be considered [9,19,21]. Recently the availability of long-circulating liposomes, which overcomes the problem of rapid uptake by the mononuclear phagocytic system, was reported [67,68,69,70]. These improved liposomes are good tools for selective delivery of drugs to target cells by means of cell-specific ligands bound to the liposome surface [71,72]. Monoclonal antibodies against cancer cells have been ligated to the surface of long-circulating (containing monosialoganglioside GM1 or phosphatidylinositol) or conventional liposomes [72]. The antibody-bearing liposomes exhibit a prolonged circulation time in the blood and increased accumulation in implanted tumors or long cancer cells in vitro [68,72]. It is claimed that extravasation of liposomes containing drug in the interstitial space around the tumor cells is related on the one hand to their long circulation time in blood and on the other hand to the increased permeability of the endothelial barrier in the newly vascularized tumors. Indeed the liposomes penetrate organs with fenestrated (i.e., liver, spleen) or deranged capillary endothelium in organs with inflammation (tumors and infections) [73]. The uptake of liposomes in infected tissues is so efficient that this effect has been used for scintigraphic imaging of deep-seated infection [74]. This effect is also utilized clinically today for the treatment of various infectious diseases [75]. However, the uptake of liposomes into tumors is not as specific as that in deep-seated infections. But the ultrastructural data concerning the extravasation of the liposomes are missing, and the

Figure 5 Tracer binding and transport in myocardial capillaries.

mechanisms by which they leave the circulation and accumulate around tumor cells is largely unknown.

Albumin, a natural molecule, was also used as a drug carrier. This molecule has the advantage of being hydrosoluble, nontoxic, and biodegradable, and because of its structure it may hold many molecules of the active drug. Bovine serum albumin nanospheres or magnetically responsive albumin microspheres coated with a specific antibody have shown encouraging results [63,76,77]. By using a directed magnetic field in the target area, it has been demonstrated in animals that it is possible to trap the particles and achieve enhanced drug delivery, although the problems of poorly perfused tumor tissue remains to be addressed. Recently, electron microscopy revealed that adriamycin-associated magnetic albumin microspheres traverse the vascular endothelium of the target tissue after 2 hours of intravenous administration in rats [76]. Microspheres are transported to the basement membrane by a process similar to transcytosis. Thus, a depot of particles is formed at the target site, which can release drugs at a controllable rate. It is possible that in the target area immune and inflammatory mechanisms become operative locally, which may also lead to beneficial effects for the host. The results obtained in small animals will soon lead to human applications.

To obtain a suitable carrier, the endogenous biological transport system must be used. Thus, the uptake of the modified plasma LDL by the scavenger receptors present on macrophages was considered by Nicholas et al. [78]. The authors have incorporated therapeutic compounds within native and acetylated LDL. The experiments showed that the parasite-infected macrophages take up the delivered drug via the scavenger receptor pathway and that the acetylated LDL is more efficient than the native LDL or the free drug.

The Fc receptor–mediated transcytosis mechanism across epithelial barriers was successfully used by Patel et al. [79]. They showed that coating drug-containing liposomes with the appropriate IgG enhances by 18-fold their transport across epithelial barriers (yolk sac endoderm and gut enterocytes).

During transcytosis the IgG-coated liposomes escape the lysosomal digestion and reach the circulation unmodified. The same strategy was used for the delivery of therapeutic drugs to the brain. Here, specialized capillary endothelial cells joined by highly restrictive junctions form a continuous wall, the blood-brain barrier (BBB), which ensures the homeostasis of this organ. No known paracellular pathway exists for peptide or drug transport through the BBB. Therefore, to gain access to brain interstitium, either receptor-mediated or absorptive-mediated transcytosis should be considered. Recent studies by Friden et al. [80] show that a monoclonal antibody OX-26 to the transferrin receptor is an efficient carrier for transporting drugs. The high density of transferrin receptors on brain endothelial cells as well as the ability of these receptors to transport molecules across the BBB by receptor-mediated transcytosis allowed the authors to demonstrate a preferential accumulation of radiolabeled nonlipophilic drug methotrexate in the brain parenchyma when the drug is conjugated to OX-26. This carrier system, which proved to have organ selectivity, could be used in the future for a wide range of compounds. Exploiting the cellular mechanism, namely, the transport of iron across the capillary endothelial cells, the authors succeeded in showing that nerve growth factor (NGF) conjugated with the antitransferrin receptor OX-26 cross the BBB after peripheral injection and enhance the survival of cholinergic neurons [90]. Both immunohistochemical and capillary depletion experiments demonstrated that biological active conjugates OX-26–EGF or OX-26–drug cross the endothelial barrier as a result of its interaction with the transferrin receptor. These encouraging results may prove useful in the treatment of neurological disorders dependent on invasive neurological procedures. Preliminary studies suggest that mildly cationized rat serum albumin and avidin, which undergo absorptive-mediated transcytosis through BBB, may serve as a stable brain drug transport carrier [81,82]. The interaction of cationic substances with negative charges present on the surface of brain endothelial cells triggers absorptive-mediated transcytosis through BBB, so that these cationic molecules could be highly efficient drug delivery carriers for the brain tissue.

In addition to the polypeptidic vectors used for the transendothelial transport of therapeutic drugs, adenovirus vectors were used recently to deliver foreign genes and promoter elements to cells of the nervous system. This method was successfully used by LeGall LaSalle et al. [91] in both in vitro and in vivo experiments. A replication-deficient adenoviral vector that contained a reporter gene encoding β-galactosidase was shown to infect rat nerve cells. Both sympathetic neurons and astrocytes in culture as well as in rat hippocampus and substantia nigra expressed β-galactosidase activity after adenoviral inoculations. The use of adenovirus vectors provides a method for the study of the function and regulation of cloned genes as well as for gene therapy.

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3

Enzyme-Albumin Conjugates: Advantages and Problems with Targeting

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I. INTRODUCTION

In the 1960s and 1970s it was believed that while enzymes possessed many outstanding properties that might make them excellent drugs (e.g., high specificity, low toxicity, ready degradation), a set of serious limitations meant that they would not readily make it to the list of common pharmaceutical agents. The problem areas included:

1. Availability of enzymes in pure form
2. Stability of the enzymes, especially in an appropriate pharmaceutical form
3. Immune reactivity of the enzyme preparation
4. Targeting of the enzyme to specific sites of action
5. Lack of suitable animal models for testing enzymes as drugs

The discipline has progressed considerably since then, although some of the limitations (immunogenicity and targeting) still exist. The advent of recombinant DNA technology, cloning, and wide-scale enzyme purification have basically solved the first problem. Our laboratory and several others [21–28] have demonstrated clearly that enzymes can be modified so as to dramatically improve on their resistance to biodegradation or stability at 37°C or in the face of a host of different plasma proteases. The problem of animal models has also been partly

alleviated with the advent of transgenic animal models of disease, at least in the growing number of cases where the disease gene has been identified and cloned (e.g., familial hypercholesterolemia, Duchenne muscular dystrophy, and cystic fibrosis).

By 1990 there were very few biologically active molecules—either small peptides or larger protein molecules such as enzymes—in common use as therapeutic agents. Insulin, of course (including recombinant human insulin), has been in use for the treatment of Type 1 diabetes, and tissue plasminogen activating factor was recently introduced as the first major new therapeutic agent produced by recombinant DNA technology. Plasma proteins, including especially the clotting agents and molecules such as albumin, had earlier been introduced for the treatment of clotting disorders or as plasma expanders, often as a function of plasma components rather than as specific purified proteins. Earlier in the century, the enzyme pepsin had been used to dress wounds, and of course digestive enzymes have been used for some time for pancreatic enzyme insufficiencies. More recently, streptokinase has been widely used for the treatment of myocardial infarction or coronary blood vessel blockage and recombinant human growth factor has been observed in widespread clinical trials, with significant success in the treatment of growth retardation or dwarfism.

The list of biologically active peptides that might be considered as potentially interesting drugs is large and growing, already numbering in the hundreds. These include a host of specific enzymes that have been identified with specific inherited enzyme-deficiency diseases and tens and probably hundreds of different growth factors that will stimulate growth or regrowth of tissue. One of the most exciting of these might be a molecule from a family of nerve growth factors, which stimulate otherwise quiescent nerve cells or perhaps damaged ones to regenerate. Then there are literally hundreds of newly discovered cell-to-cell mediators that produce medically relevant reactions such as cytokine-induced killing of cancer cells.

II. LIMITATIONS OF ENZYME/PEPTIDE THERAPY

While we learn more about the normal physiological role of many of these peptides and the potential they show as therapeutic agents, we are only starting to scratch the surface of how to make use of them as therapeutic agents. Some of the key questions at this stage include:

1. How is the peptide to be administered, and how will it reach its desired site of action?
2. How is the time over which the peptide is to be active regulated?
3. Will the peptide, as an exogenous or at least exogenously administered protein, be immunogenic?

These are questions that we are only now beginning to grapple with in a serious or concerted manner. As a matter of interest, many of the problems associated with the delivery of exogenous peptides will be reencountered as we move into the area of gene therapy. Even as we develop techniques of gene therapy, there will remain the problem of directing the genes to specific sites of action.

A key solution to the use of bioactive peptides is the notion of carriers or drug-delivery systems: mechanisms to package the peptides in a concentrated and protected manner and deliver them to particular sites of action or alternatively release the peptides at specific rates over time. The range of carrier or delivery systems seems almost limitless, and the subject has been reviewed on many occasions (e.g., Ref. 21). The most commonly considered vehicles are liposomes or lipid vesicles encapsulating an aqueous environment, with water-soluble or lipid-soluble drugs being incorporated into the water or lipid phases. The composition of the lipid may be altered to change the loading factors or trapped volumes, and the chemical composition may be chosen in a way as to control the extent of interaction with specific sites. Targeting agents such as site-specific antibodies can be added to target the liposomes. Polyethylene glycol (PEG) may be conjugated to the lipid surface to alter the particles; pharmacokinetics, or the surface charge on the liposome may be altered either chemically or biochemically to change the manner in which the particles are recognized.

The following molecules or carriers have been shown to function in a variety of forms as drug/peptide carriers or targeting systems:

Semipermeable microcapsules	Hepatocytes
Liposomes	Nanoparticles
Antibodies	Lipid vesicles
Fab′ fractions	Dextran
Emulsified lipid	Albumin
DNA	Denatured albumin
Polylysine	Lipoproteins
Fibrinogen	Lectins
Collagen	Polyethylene glycol
Fibroblasts	Polyglutamic acid

III. ENZYME-ALBUMIN CONJUGATES

Our laboratory has for a number of years championed the use of the plasma protein albumin as an effective carrier to protect enzymes for use in enzyme-replacement therapy. Albumin is the most abundant plasma protein and has a circulation half-life of as much as 60–72 hours. It has both hydrophilic and

hydrophobic binding sites and has been shown to function physiologically as a carrier of small (often hydrophobic or amphiphilic) peptides. We have demonstrated for a wide range of enzymes that covalently attaching the enzyme to an excess of albumin using a variety of different cross-linking agents gives the enzyme: (1) resistance to biodegradation, (2) immunological nonreactivity, (3) an increasingly long circulation time in the plasma, and (4) the potential for site-specific targeting using either antibodies or specific ligands such as hormones.

The enzymes that we have conjugated with albumin include:

Uricase	Phenylalanine, ammonia lyase
Catalase	Superoxide dismutase (yeast)
α-1,4-Glucosidase	L-Asparaginase
Cholesteryl esterase	Glucose-6-phosphatase
Superoxide dismutase (Cu,Zn)	Superoxide dismutase (Mn)

A number of different ligands or targeting agents have also been conjugated to albumin-enzyme conjugates to direct them to specific cells or tissues [25–27]. Many of these experiments have been performed in tissue culture conditions so that the efficacy of in vivo targeting remains to be established. We know, however, that the endothelial surface remains a formidable barrier for the movement of specific peptides from the site of administration, often the circulation, to underlying target tissues. Some of the ligands are:

Insulin
Secretin
Apo B (from LDL)
Monoclonal antibodies to pancreatic tumor cells
Polyclonal antibodies to either hepatocytes or fibroblasts

The following paragraphs will highlight some of the data from earlier experiments using enzyme-albumin and enzyme-albumin-targeting agent conjugates. First we describe some of these properties. Then we describe some preliminary experiments in terms of the endothelial barrier and how peptides or peptide/carrier complexes might cross the endothelial barrier to arrive at underlying target tissue. The mechanisms and problems associated with this permeability barrier are also the subject of another chapter in this volume.

Table 1 demonstrates the manner in which conjugating an enzyme to albumin renders it significantly resistant to biodegradation either by temperature or by proteolysis. This had been demonstrated as early as the 1950s for the case of immobilized enzymes on solid matrices but appears equally so when the enzyme is immobilized in soluble form [22,23]. We do not yet have similar data for the biological activity of small peptides and their resistance to biodegradation, although given the fact that biologically active proteins prefer a concentrated

Table 1 Enzyme Resistance to Biodegradation

Enzyme preparation	% Activity remaining following 4 hr at 37°C + 5 units trypsin
α-Glucosidase	10
α-Glucosidase/Albumin (1:10)	85
Superoxide dismutase (SOD)	50
SOD/Albumin	95
SOD/Albumin-antibody	95 (60)[a]
Cholesteryl esterase (CE)	20
CE/Albumin-insulin	75 (45)[a]

[a]Values in parentheses refer to the retention of the ligand or targeting activity (antibody or insulin).

protein solution, it is likely that this will remain true for smaller peptides as well. Furthermore, we have evidence from our work with insulin as a targeting agent (see below and Ref. 27) that such ligands may remain active even in a conjugated form.

Table 2 documents the altered immunogenicity of the enzymes conjugated with an excess of albumin. It is clear that in order to render the enzyme non-immunogenic, the albumin must be homologous in origin and that a certain number of albumin molecules are needed to reduce the antigenic nature of the enzyme molecule. Our evidence previously concluded that the albumin was effectively masking the antigenic sites on the enzyme as a result of the cross-linking. Other experiments also pointed to the possibility that the albumin was in fact functioning to induce tolerance to the attached protein, effectively acting

Table 2 Immunogenicity of Albumin-Conjugated Enzymes

Enzyme preparation	Immune reaction[a]
α-Glucosidase	+++
α-Glucosidase/Albumin (1:10)	–
Superoxide dismutase (SOD)	+++
SOD/Albumin (1:5)	+
SOD/Albumin (1:10)	–
Uricase	+++
Uricase/Albumin (1:10)	–

[a]+++ represents a strong immune reaction in mice; – represents no immune reactivity.

as a hapten. We have not resolved the question of mechanism in terms of the nonimmunogenicity of the conjugated enzyme. Suffice it to say that we have been able to reduce or obliterate the antigenicity of every enzyme we have attempted to conjugate with albumin.

Tables 3 and 4 show some of the pharmacokinetic data for a number of enzymes in their free and conjugated forms with albumin. A number of points are clear. When enzymes are conjugated with an excess of homologous albumin (rat albumin for most of the results described), their rates of removal from the circulation and their sites of uptake resemble more that of albumin than that of the native enzyme. Included in Table 4 is some data relative to our attempts to target a number of enzyme α-glucosidase and cholesteryl esterase) albumin conjugates using specific ligands such as insulin and apoB derived from LDL, in the first case attempting to target the enzyme to muscle and in the second to liver. It seems clear from the data that targeting material to the liver is likely to be straightforward, whereas targeting to other tissue such as muscle may be much more difficult.

The issue of targeting is far more complex than simply showing a specific affinity of a particular carrier complex with a specific cell or even the ability of the cell to take up the complex and have the drug or peptide activity expressed. Here we must seek to understand how the complex reaches the site of action from the site of administration. Except for cases where this involves local or topical administration, it invariably implies the circulation or blood stream as a pathway and therefore the endothelial cell membrane or layer as an important barrier. A second important obstacle is really a negative barrier, i.e., the reticuloendothelial system (RES), including macrophages, whose function it is to remove unfamiliar

Table 3 Plasma Clearance of Albumin-Conjugated Enzymes

Enzyme preparation	Circulation half-life (min)
α-Glucosidase	40
α-Glucosidase/Albumin	260
α-Glucosidase/Albumin/Insulin	180
Superoxide dismutase (SOD)	6
SOD/Catalase conjugates	160
SOD/SOD conjugates	140
SOD/Albumin (1:5)	240
Growth hormone (porcine)	15
Growth hormone/Albumin	150

^{125}I-labeled enzyme (or growth hormone) preparations were injected into rats, and plasma levels of the ^{125}I protein were followed as a function of time.

Table 4 Tissue Deposition of Intravenously Injected Enzymes and Enzyme/Albumin Conjugates

	Tissue levels (%)[a]					
Enzyme preparation	Blood	Liver	Spleen	Kidney/ Urine	Muscle	Other
SOD (5 min)	48	10	2	22	—	28
SOD (60 min)	5	15	2	55	—	23
SOD/Albumin (5 min)	80	4	1	1	—	14
SOD/Albumin (120 min)	60	6	1	2	—	31
α-Glucosidase[b]	15	55	4	3	2	21
α-Glucosidase/Albumin	65	18	2	1	1	13
α-Glucosidase/Insulin	40	30	3	1	4	22

[a]Tissue values are corrected for blood content and represent percent of the injected ^{125}I dose.
[b]All glucosidase analysis times are 180 min.

material from the circulation. Our original intention was to use the common plasma protein albumin as a means of avoiding the RES. Tables 5 and 6, and Figure 1 describe some of our preliminary attempts to study the endothelial barrier and determine how complex drugs/peptide systems might be designed to pass the barrier in a specific manner so as to arrive at target sites on the other side.

Before initiating this work, we started from a hypothesis derived from the work of Palade and the Simionescus [17,29–33] and described by us in a review article in 1984 [21]. The hypothesis suggested that the process of receptor-mediated transcytosis (Fig. 1) was likely responsible for the movement of specific biological macromolecules across the endothelial barrier. A significant controversy exists with respect to the relative importance of the transcellular and paracellular pathways [1,14,15]. The experiments described in Tables 5 and 6 indicate that both pathways are plausible but more importantly that the process of transendothelial transport may be receptor dependent.

The experiments documented in Tables 5 and 6 use confluent monolayers of pure bovine aorta endothelial cells that were grown in tissue culture petri dishes, then seeded at high concentration on collagen-coated polycarbonate filters (pore diameter = 0.8 μm). The filters were fitted onto millipore filter holders placed in a buffer compartment with separate access to both sides of the filter and thus both the apical and basal surfaces of the endothelial cell monolayer. The asymmetry of the monolayer and the integrity of the tight junctions had previously been assessed using antifactor eight antibodies as a marker for the plasma or basal surface of the cells and transmembrane conductivity as a measure of the resistance.

Table 5 Endothelial Cell Monolayer Permeability

Peptide/Enzyme preparation	% Equilibration[a] in 4 hr
5000 kDa peptide	0–2
Superoxide dismutase (SOD)	0–2
SOD/Albumin	2.5
Insulin	17.5
SOD/Albumin/Insulin	15
SOD/Albumin/Insulin + excess insulin (50×)	5
LDL	20
Cholesteryl ester (CE)	0–2
ApoB/CE complex	15
Glucose	60

[a]% Equilibration refers to the extent of equilibration of ^{125}I concentration between the apical and basal solutions. For protein complexes, the enzyme is the iodinated (^{125}I) molecule. For glucose, the label is 3-H.

Table 5 verifies the low permeability of the monolayer to a number of proteins ranging in molecular weight from a 5000 kDa random synthetic peptide with primarily hydrophilic amino acids to a 600,000 kDa conjugate of albumin and superoxide dismutase with an average molar ratio of approximately 10:1. The movement of these molecules across the monolayer over a 4-hour period is extremely slow and only slightly above background. Insulin and LDL cross the monolayer quite effectively and in a concentration-dependent manner. These experiments verify earlier work from King's laboratory [2,8]. Furthermore, when

Table 6 Endothelial Cell Monolayer Permeability Transcellular and Paracellular Paths

Permeant measured	Conditions	% Equilibration in 4 hr
Neutrophils	—	0
Neutrophils	fMLP	65
SOD/Albumin	—	0–2
SOD/Albumin	Neutrophils	0–2
SOD/Albumin	Neutrophils/fMLP	45
LDL	—	20
LDL + cell LDL (50×)	—	2.5
LDL	Neutrophils/fMLP	55
LDL + cold LDL (50×)	Neutrophils/fMLP	50

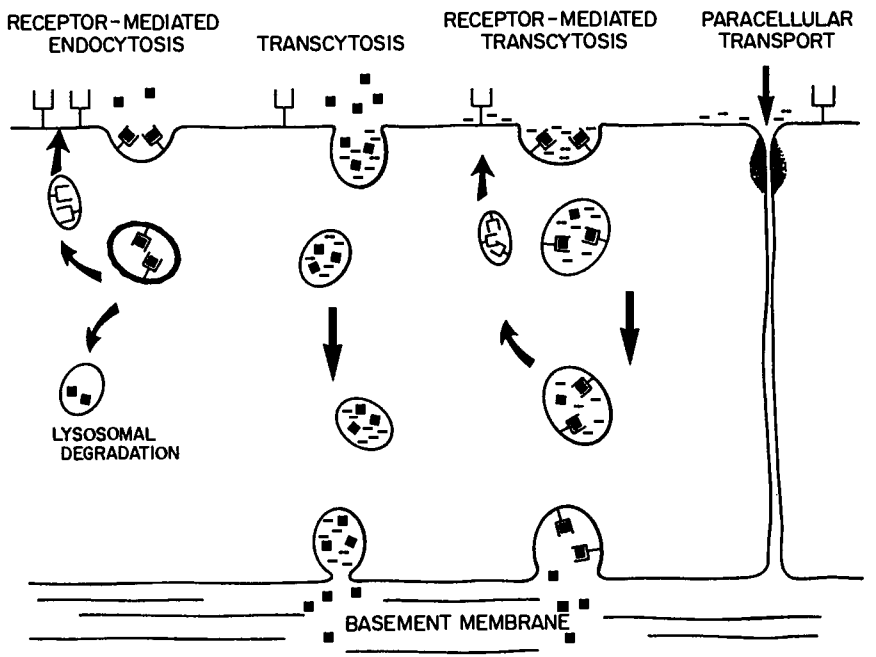

Figure 1 Hypothetical process of receptor-mediated transcytosis, including other potential routes across the layer.

insulin or ApoB are linked to either albumin, self conjugates of albumin, or SOD-albumin conjugates, the proteins appear to piggyback and cross the barrier utilizing the ligand specific transport system. The transport is determined by measuring the movement of ^{125}I-labeled protein from one compartment to the other (usually basal to apical). The ligand dependence or specificity of the process is further verified by demonstrating that a 50-fold excess of nonconjugated ligand (either insulin or ApoB) effectively competes for and inhibits the transendothelial movement of the protein complex, which included conjugated ligand. These data argue in favor of the process of receptor-mediated transcytosis and the movement of the ligand-protein complex across the cell monolayer via a transcellular pathway.

Table 6 shows data that attempt to distinguish between the transcellular and paracellular (between cells) pathways for peptide movement across endothelial cell monolayers. In these experiments we describe the movement of proteins in various forms across the monolayer in the presence and absence of an active

paracellular pathway created by the movement of stimulated neutrophils across the monolayer. In this case the neutrophils are thought to cross the endothelial barrier through an active "opening" of the tight junction or paracellular pathway by some protease-based system. A number of points are evident from this table:

1. Neutrophils only cross the monolayer from the basal surface and increase the permeability of the barrier in response to a stimulant (in this case the presence of a chemoattractant, fMLP) in the chamber facing the apical cell surface.
2. Biological macromolecules (albumin or albumin-enzyme conjugates both with and without conjugated ligands) cross the endothelial cell monolayer following the movement of neutrophils but also independent of the neutrophils and without any receptor dependency.

We conclude from Tables 5 and 6 that biological macromolecules involving specific ligands can cross the endothelial monolayer barrier in at least two ways. They can cross via a transcellular pathway that we and others have termed receptor-mediated transcytosis and which we believe to be very specific and very directed. They can also cross the barrier by a paracellular pathway involving neutrophil-dependent opening of the tight junction between endothelial cells and an increase in conductance or permeability across the cell monolayer. From our experiments we would anticipate that this is a much less specific pathway than the one described above and may be more responsible in vivo for the bulk movement of fluids, including peptides across the endothelial barrier from the circulation to underlying tissue.

IV. CONCLUSIONS

In considering the use of small peptides and enzymes as drugs, much more attention must be paid to the issues of protein stability and the ability to target or direct the drug to appropriate sites of action. This is perhaps much more crucial than for small hydrophobic or hydrophilic drug molecules, which enjoy ready access to many targets following oral administration. Albumin, which in itself is a natural carrier in the plasma, offers certain advantages in terms of protecting the attached peptide or enzyme from biodegradation and potential immune reactivity and allows the drug to have a prolonged circulation time. The issue of enzyme/peptide targeting remains an important limitation to the common use of enzymes as drugs. In considering the notion of receptor-mediated transcytosis and how endogenous peptides, proteins, and enzymes move around the body via the circulation, we may learn something about how to design an appropriate delivery

system (see, e.g., Refs. 2,9,10). Finally, in considering the notion of enzyme/peptide therapy, we have to be cognizant of the issue of pharmaceutical feasibility. Is this a therapeutic approach that we expect to be limited to a sophisticated university hospital setting, or is it something that we expect to use in the field—in a patient's home or the physician's office? This issue will be critical in the design of enzyme/peptide delivery systems.

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4

Acyl Enzymes as Prodrugs

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I. INTRODUCTION

Several pharmaceutical enzymes belong to the group of serine-histidine estero-proteolytic enzymes (serine proteases), which display their catalytic activity with the aid of an especially reactive serine residue, whose β-hydroxyl group forms a covalent bond with the substrate molecule. This reaction takes place by cooperation with the imidazole base of histidine. The specificity of the enzymes is achieved by the characteristic structure of their substrate-binding centers, which in these proteases are built according to the same principle. They consist of a hydrophobic slit formed by apolar side chains of amino acids and a dissociated side chain–located carboxyl group of an aspartic acid residue at the bottom.

The serine proteases exhibit distinct specificities for proteolysis of large substrates and for esterolytic hydrolysis of small substrates. The hydrolysis of the substrate takes place in the following kinetically distinct steps (Fig. 1):

The reaction of the enzyme (E) with the substrate (S) comprises the formation of an adsorptive complex (E·S).
A covalent intermediate, the acyl enzyme (E-S′), liberates the first product P_1.
The deacylation of the acyl-enzyme finally releases the product P_2.

Rather interesting conclusions can be drawn from an examination of the serine protease–catalyzed hydrolysis of a series of esters or anilides of basic substituted benzoic acids and amidinophenyl esters of aromatic carboxylic acids [1–10]. They react with the serine proteases like a substrate by acylation of the serine residue of the active center and by simultaneous release of the alcoholic

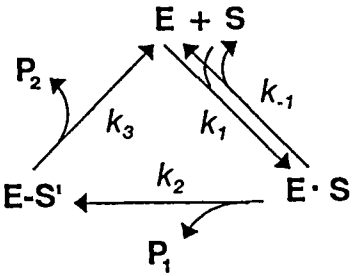

Figure 1 Reaction pathways for catalysis by serine proteases.

component (P1) Since the acyl enzyme hydrolyzes to the free enzyme at extremely low velocity only, the absolute concentration of the active enzyme can be determined by spectrophotometric measurement of P_1. Therefore, several of these synthetic substrates, especially the nitrophenyl esters of guanidino-benzoic and amidino-benzoic acid, serve as excellent "active-site titrans" for the enzymes [11–15].

Covalent intermediates in serine protease catalysis have played a significant and historical role in our understanding of enzyme mechanism. During catalysis the substrate will pass through at least two covalent intermediates. The tetrahedral adduct is formed before generation of the acyl enzyme. Trapping of covalent acyl enzymes from specific substrates is very difficult since in this substrate type the rate of deacylation is much higher than the rate of acylation. For activated ester substrates, the overall rate-determining step is hydrolysis of the acyl enzyme, and the acyl intermediate can be trapped by using low pH and low temperature [16]. The first comparatively stable acyl derivatives of trypsin and related enzymes were obtained by acylation of the enzyme with the pseudo-substrates guanidinobenzoates and amidinobenzoates [1,6,7].

During the development of synthetic, low molecular weight (LMW) inhibitors of trypsinlike serine proteinases, we found a series of 4-amidinophenyl benzoates to act as ester substrates of trypsin and the hemostaseologically interesting serine proteases in blood, thrombin, and plasmin [17,18]. In contrast to other ester substrates, the amidinophenyl benzoates have a cationic center in the leaving group. Therefore, with respect to their structure and kinetic characteristics, they were named "inverse substrates" [19,20]. In principle, their hydrolysis by trypsinlike enzymes (E) proceeds like that of other substrates (S). This comprises the formation of the noncovalent Michaelis complex (E·S) and a covalent intermediate (E-S), the acyl enzyme. The first product P_1 liberated is *p*-amidinophenol, and P_2 (benzoic acid) is finally released by deacylation.

Figure 2 shows hydrolysis of an ester of *p*-amidinophenol by trypsinlike enzymes. Deacylation is much slower than acylation, and accumulation of the acyl enzyme occurs. For preparation of a wide range of acyl derivatives of serine proteases, this type of substrate has become increasingly important.

Because of their high affinity and their ability to form inactive acyl enzymes, these synthetic substrates represent temporary inhibitors. However, the *p*-amidinophenol esters could be used for the pharmacological control of serine proteases to a limited extent only because of the transience of their inhibitory action.

More recently, these esters increased in significance for the preparation of stable acyl enzymes, which regain their activity by deacylation and, accordingly, have prodrug characteristics. Therefore, we have continued our investigations into amidinophenyl benzoates in order to prepare acylated serine proteases of pharmacological interest (Table 1).

II. BIOCHEMISTRY

For preparation of a wide range of acyl derivatives of trypsinlike enzymes, the 4-amidinophenyl benzoates proved appropriate, and using these tools preparation of acyl enzymes is relatively easy. In the first step the enzyme is acylated in the presence of excessive ester concentrations. Thereafter deacylation is stopped by lowering pH and temperature. Ultimately, the acyl enzyme is isolated from the

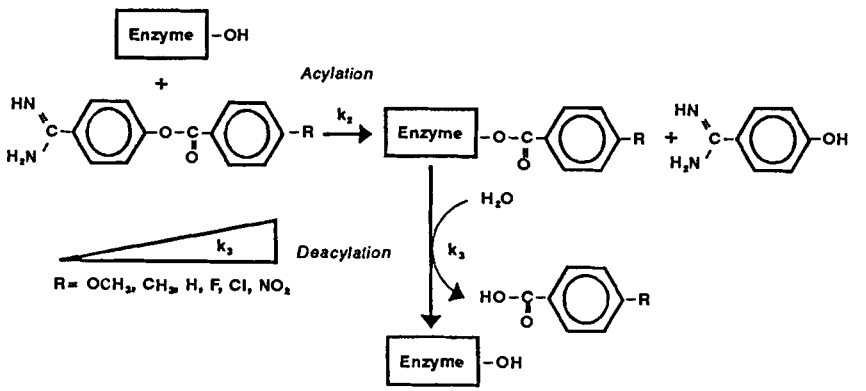

Figure 2 Schematic representation of acylation of serine proteases by amidinophenyl benzoates: deacylation rate (k_3) depends on benzoic acid substitution (R).

Table 1 Acylation of Selected Pharmacologically Important Serine Proteases

Enzyme	Effect	Target	Ref.
Thrombin	Fibrinogen coagulation	Hemostasis	6,33
Batroxobin	Fibrinogen coagulation	Defibrinogenation	40,41
Plasmin	Fibrinolysis	Thrombolysis	21,22
Plasmin-streptokinase-activator complex	Plasminogen activation	Thrombolysis	23–25
Urokinase	Plasminogen activation	Thrombolysis	30,31
Tissue-type plasminogen activator (t-PA)	Plasminogen activation	Thrombolysis	32
Kallikrein	Kinin liberation	Kinin affects vasodilation	41,42

excess ester and the liberated amidinophenol. Dialysis, gel filtration, and ultrafiltration are suitable methods. Solutions of acyl enzymes or lyophilized preparations are frozen and stored at low temperature. On deacylation the enzyme is reactivated.

Studies on acylation and deacylation of trypsinlike enzymes by *p*-amidinophenyl esters of substituted benzoic acids have shown that the rate of deacylation is influenced by the benzoic acid substituent. There is a linear correlation between the rate of deacylation and the inductive effect of the substituent (Fig. 3). This is also true of all trypsinlike serine proteases tested. However, the degree of the deacylation rate varied greatly in different members of the trypsin family, although the same acyl group had been introduced into the active site. The constants for reactivation of various benzoyl enzymes are given in Table 2. In contrast to tissue plasminogen activator and trypsin, plasmin and thrombin remained in the acylated state for a prolonged period of time. Of course, the half-life of reactivation of these enzymes may be shortened or prolonged by introduction of the corresponding substituted benzoic acid residues. That means for every enzyme a wide variety of acyl derivatives with extremely different deacylation can be prepared, so that the design of acyl derivatives with desired deacylation kinetics is feasible.

Of special interest is the reaction of an acyl enzyme with active-site–directed inhibitors in comparison with the free enzyme. Therefore, we studied the hydrolysis of the substrate Bz-Arg-pNA by trypsin and benzoyl-trypsin in the presence of the naturally occurring inhibitor aprotinin [21]. The substrate is cleft

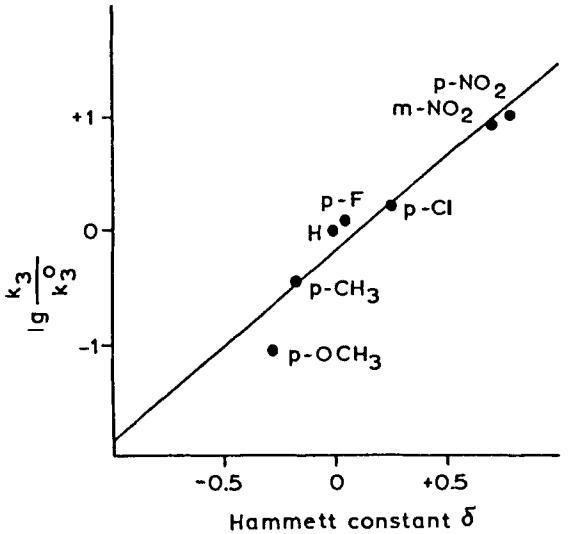

Figure 3 Correlation between rate of deacylation (k_3) and Hammett constant (σ) of acyl-trypsin. (From Ref. 6.)

Table 2 Half-Time (min) of Reactivation of Serine Proteinases Acylated with Substituted Benzoic Acids at pH 7.5 and 37°C

Enzyme–O–OC–R + H_2O → Enzyme + HOOC–R

	R =						
	OCH_3	CH_3	H	F	Cl	CN	NO_2
Trypsin (bovine)	53.3	18.7	6.4	5.2	3.8	—	0.6
Thrombin (bovine)	433.0	301.0	239.0	116.0	35.0	—	6.0
Batroxobin (bothrops)	1155.0	239.0	55.0	50.0	28 .0	17.0	16.0
Plasmin (human)	72.2	49.5	21.7	22.4	8.9	—	2.3
t-PA (recombinant)	10.7	9.0	2.6	2.4	1.2	0.7	0.7
Urokinase (LMW)	3.0	2.3	2.9	1.0	—	—	—
Urokinase (HMW) (pancreatic)	36.5	—	8.0	—	—	—	—
Kallikrein	289.0	178.0	54.0	14.1	10.3	6.5	2.8

by the enzyme at a constant rate throughout the observation time. The addition of aprotinin causes rapid inhibition of trypsin, so that the enzyme is completely inactivated after 15 minutes. When, instead of trypsin, the same amount of benzoyl-trypsin is used, the enzyme is slowly generated and 100% of enzyme activity is reactivated after 30 minutes. In the presence of the inhibitor, moderate but relatively constant enzyme activity occurs for a prolonged period of time. Such course of enzyme activity can be obtained only by continuous enzyme supply (Fig. 4).

III. PHARMACOLOGY

Acyl enzymes have recently become objects of pharmacological interest as potential prodrugs. This is best illustrated by the following propositions:

The acyl enzyme is an inert form of the enzyme for which—by an intramolecular mechanism—the active enzyme is generated. Therefore, the reversibly acylated enzyme could be used as a delivery system for the active enzyme whose action is both delayed and prolonged in plasma (depot effect).

Interactions of the serine proteases with active-site–directed inhibitors are blocked by acylation. Therefore, in contrast to the active enzyme, the acyl enzyme is protected from being inactivated in plasma by rapidly acting inhibitors (protection against inactivation).

If the elimination of the enzyme proceeds via binding to plasma inhibitors, it becomes evident that temporary inactivation of the active center blocks at

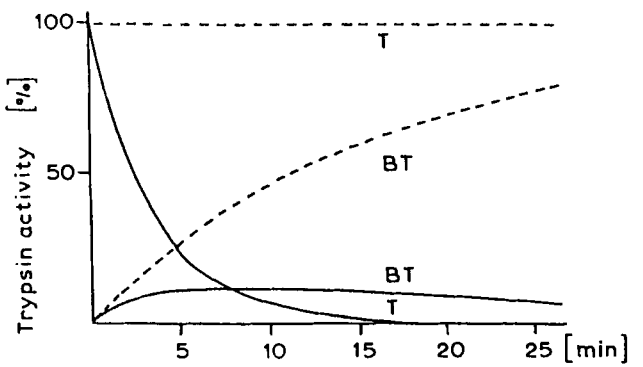

Figure 4 Reaction of trypsin (T) and benzoyl-trypsin (BT) (0.1 μmol/L) with synthetic substrate (Bz-Arg-pNA) in the absence (dotted line) or presence (solid line) of the inhibitor aprotinin. (From Ref. 21.)

least one major pathway of physiological clearance of the serine proteases (prolongation of elimination).

Summarizing, an acyl enzyme behaves like a prodrug in vivo that is slowly reactivated in the circulation and is not inactivated by plasma inhibitors. Thus, it shows a higher and longer-lasting in vivo activity than the corresponding free enzyme. The potential efficiency of several acyl enzymes as prodrugs was the starting point for the acylation of those serine proteases that are therapeutically useful (Table 1). We dealt especially with the preparation and pharmacological characterization of acylated clotting enzymes (thrombin and batroxobin), acylated fibrinolytic enzymes (plasmin, urokinase, and tPA), and the acylated kinin-liberating enzyme kallikrein.

A. Clotting Enzymes

The reactions leading to the coagulation of blood can be divided into two major pathways: the intrinsic and the extrinsic mechanisms (Fig. 5). Factors XII, XI, IX, VIII, X, and V participate in the intrinsic pathway, while tissue factor and factors VII, X, and V participate in the extrinsic pathway. These two pathways come together with the activation of factor X. A major feature of this proposal is that the various clotting factors are present in plasma in a precursor form. When the coagulation process is triggered, these clotting factors interact with each other in a stepwise manner in which one acts as an enzyme and the other as a substrate. During these reactions, the clotting factors are converted to an activated form. Eventually, thrombin is formed, which converts fibrinogen to fibrin, representing the final step in this series of reactions. We tested the effect of the acylated clotting enzyme thrombin and batroxobin, the thrombinlike enzyme from snake venom. A further candidate for the preparation as acyl enzyme, not yet investigated, would be the clotting factor Xa, the prothrombinase.

1. Thrombin

The serine protease thrombin takes a central position in the clotting system. It splits off fibrinopeptides A and B from the amino terminal ends of the α- and β-chains of fibrinogen. The resulting fibrin monomer then undergoes polymerization to form a fibrin clot. Via activation of the clotting factors V and VIII, further thrombin is generated from prothrombin, and via activation of blood platelets and of the fibrin-stabilizing factor XIII the soluble fibrin clot is cross-linked to form a tough, insoluble fibrin polymer. Furthermore, thrombin has direct effects on vascular endothelium and mediates non-hemostatic cellular events (Fig. 6). The enzyme generally isolated from bovine plasma is used as local hemostatic agent because it stops bleeding from small vessels within a few seconds.

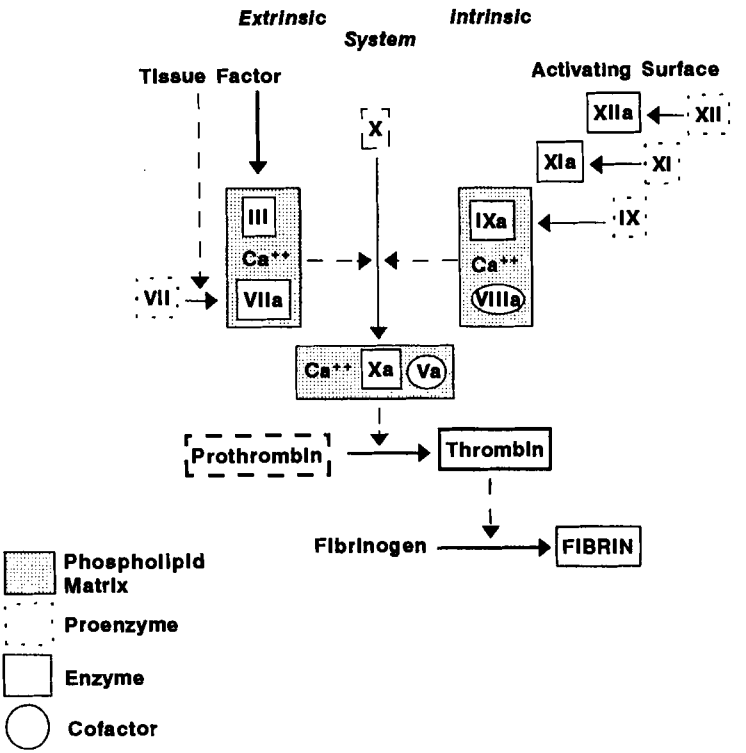

Figure 5 Scheme of the coagulation system.

To demonstrate the effect of the active-site acylated enzyme, we prepared the benzoyl derivatives of bovine thrombin [22,23]. If benzoyl-thrombin is added to blood plasma, the clotting process is retarded in accordance with the deacylation rate (Fig. 7).

The acylated enzyme is not inactivated by the progressively acting plasma-antithrombin in spite of the prolonged incubation time, as demonstrated in our previous experiments with synthetic inhibitors, which block the active center of the enzyme [24].

Of special interest would be the cellular reaction of acyl-thrombin. By incubation of platelet suspension with benzoyl thrombin, the platelet reactivity was diminished. The same phenomenon could be observed in platelet suspensions where small amounts of thrombin were added slowly [25]. Further studies on nonhemostatic reactions of acyl-thrombin have not yet been carried out.

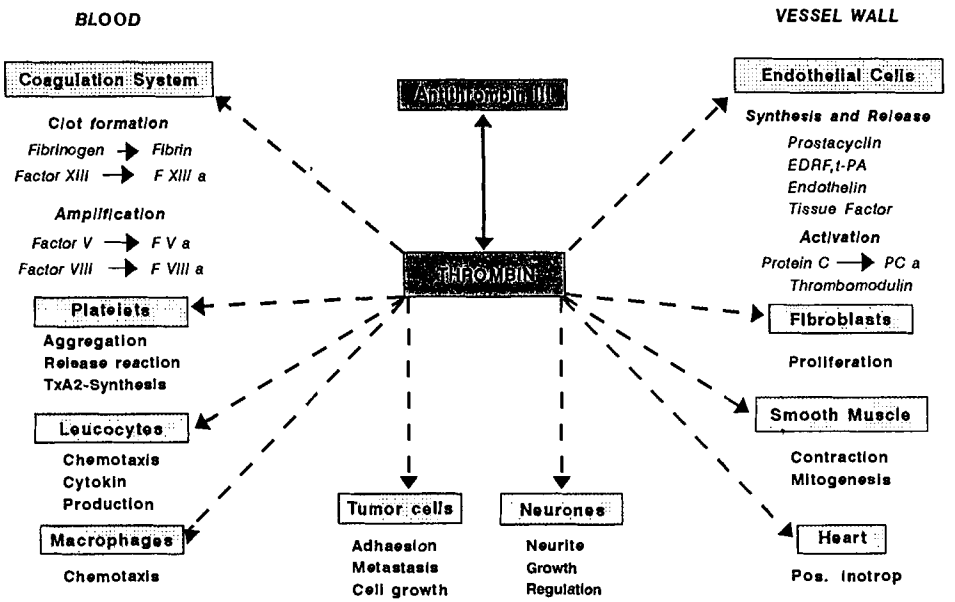

Figure 6 Scheme of thrombin reactions.

In vivo, the infusion of small amounts of thrombin (<200 NIH-U/kg/h) causes defibrinogenation. Because of the slow transformation of fibrinogen, the fibrin monomers formed are eliminated via reactive fibrinolysis and RES without precipitation of fibrin. Upon infusion of higher amounts of thrombin, these clearance mechanisms fail to offset the thrombin action, which then leads to the formation of microthrombi in the peripheral circulation. Correspondingly, the bolus injection of 1500 NIH-U thrombin/kg caused death of the experimental animals. However, a bolus injection of benzoyl-thrombin at a dose corresponding to the amount of 1500 NIH-U/kg caused defibrinogenation only. Therefore, bolus application of acyl-thrombin may initiate an infusion regimen [26].

2. Batroxobin

Batroxobin, the thrombinlike enzyme from the venom of *Bothrops moojeni*, is used for therapeutic defibrinogenation. In contrast to thrombin, which converts fibrinogen to fibrin by splitting off both fibrinopeptides A and B, batroxobin splits off only fibrinopeptide A. Des-A-fibrin monomers formed aggregate to atypical clots, which are trapped in the microcirculation and subsequently removed by secondary fibrinolysis and phagocytosis [27]. In case of insufficient fibrinolysis,

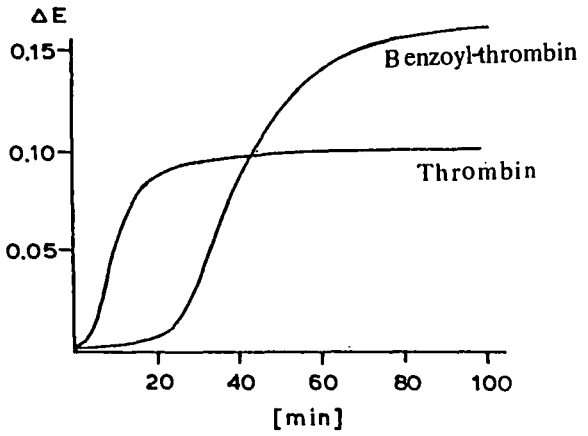

Figure 7 Course of fibrinogen clotting (measured turbidimetrically) on addition of 5 NIH-U thrombin (T) and benzoyl-thrombin (BT).

microthrombosis will become manifest. Therefore, in clinical practice the initial dose is given as a continuous i.v. infusion.

By the use of 4-amidinophenyl benzoates, acyl-batroxobins with different deacylation rates and thus with governable duration of action can be prepared. In order to clarify whether acyl-batroxobin behaves like a delivery system in vivo, the influence of batroxobin and benzoyl-batroxobin on defibrinogenation and microthrombosis was studied in rats [28,29]. The decrease in fibrinogen induced by bolus injection of batroxobin or benzoyl-batroxobin is shown in Fig. 8. Injection of batroxobin caused a dose-dependent decrease in fibrinogen, which is accompanied by transient microthrombosis. Compared to the effect of the non-acylated enzyme, fibrinogen decreased more slowly after bolus injection of an equivalent dose of benzoyl-batroxobin, and no signs of microthrombosis were detectable [30].

Our studies have shown that benzoyl-batroxobin administered as bolus injection produced an effect similar to that observed after infusion of batroxobin. Accordingly, benzoyl-batroxobin is used as a delivery system for the defibrinogenating enzyme, which can be applied as bolus injection.

B. Fibrinolytic Enzymes

The fibrinolytic system of the body, which is designed to dissolve fibrin formed in the course of blood coagulation, becomes active via enzymatically controlled

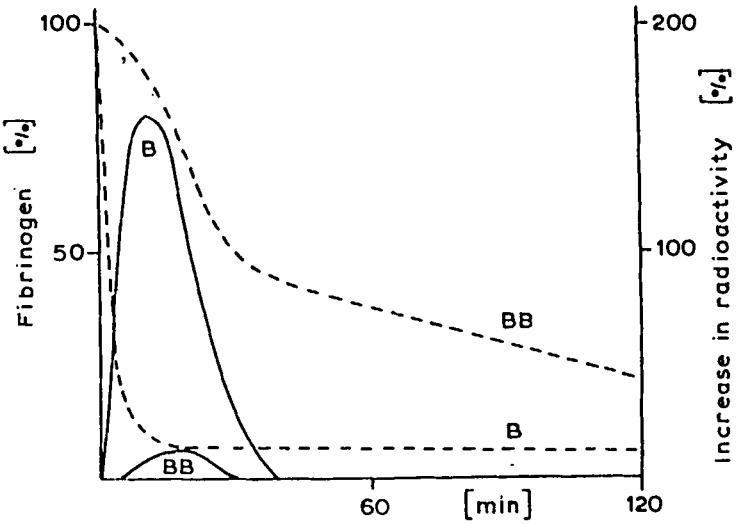

Figure 8 Content of fibrinogen in rat plasma (dotted line) and increase in radioactivity over the rat lung (solid line) after pretreatment with ^{125}I-fibrinogen and i.v. injection of 10 units of batroxobin (B) or benzoylbatroxobin (BB).

proteolytic reaction chains initiated by release or formation of activators of fibrinolysis. The largely specific thrombolytic activity is due to fixation of the activator enzymes to specific binding sites of fibrin. Here, plasminogen, fixed to the same binding sites, is activated to the fibrinolytically active plasmin, which acts directly on the substrate fibrin.

Acyl derivatives of fibrinolytic enzymes, both of plasminogen activators (tPA, uPA) and plasmin, were studied in detail.

1. Plasmin

So far, the endogenous fibrinolytic enzyme plasmin could not successfully be used in thrombolysis because upon injection, it was immediately blocked by endogenous inhibitors, particularly by α_2-antiplasmin. Plasmin became fibrinolytically effective only after consumption of these inhibitors. To obtain a fibrinolytic effect, large quantities of plasmin were required, which in turn led to hyperplasminemia. This caused proteolytic degradation of fibrinogen and several clotting factors and hence hemorrhagic complications.

The acylated enzyme does not react with the plasma inhibitors in the same way. However, it is obviously fixed to fibrin and deacylated there, so that the fibrinolytic effect remains restricted to the thrombus (Fig. 9).

In order to compare the efficiency of acyl-plasmin to that of the free enzyme, we studied the lysis of microthrombi induced in the lungs of experimental animals [31]. To obtain a rapid onset of fibrinolysis, we prepared the acylated human plasmin by incubation with the amidinophenyl ester of *p*-chlorobenzoic acid. The *p*-chlorobenzoyl-plasmin obtained is relatively rapidly deacylated in plasma ($t_{1/2}$ of deacylation = 10 min). A single administration of a relatively small quantity of acyl-plasmin was able to eliminate microthrombi. The same quantity of free plasmin remained ineffective (Fig. 10).

2. Streptokinase-Plasmin-Activator-Complex

Streptokinase and plasminogen form a complex having the characteristics of a plasminogen activator and activating plasminogen molecules not yet combined with streptokinase to plasmin. The concept of acylation of fibrinolytic enzymes

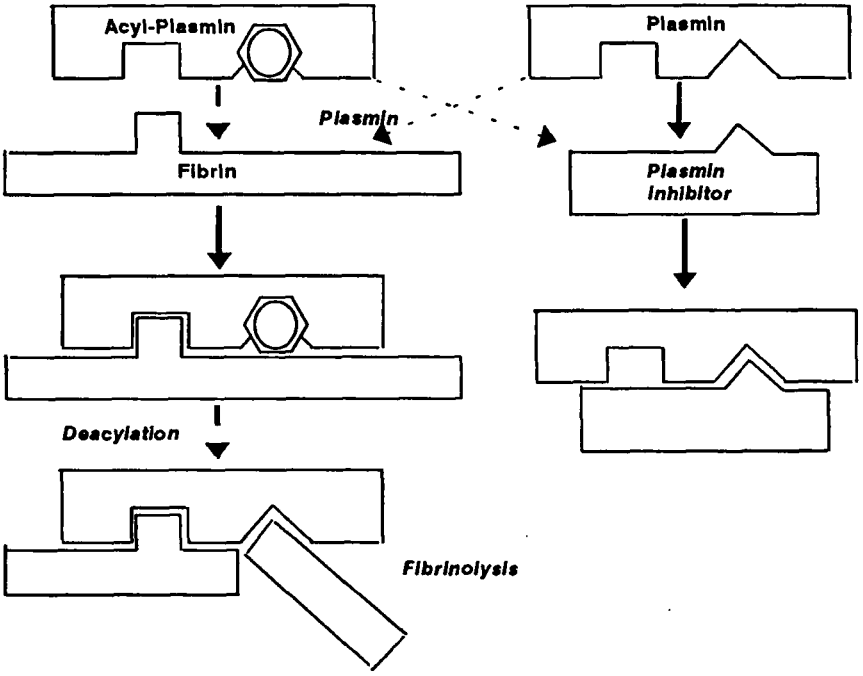

Figure 9 Scheme of reaction of acyl-plasmin.

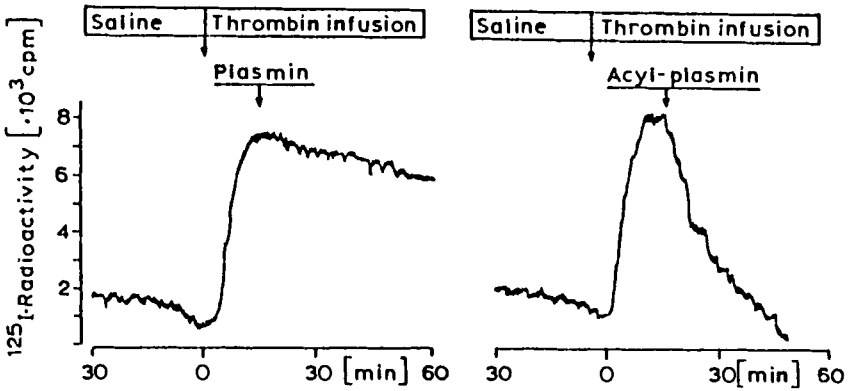

Figure 10 Influence of plasmin and chlorobenzoyl-plasmin (300 PTA-U/kg i.v.) in rats during thrombin infusion (1000 NIH-U/kg/h). After the animals had received ^{125}I-fibrinogen, microthrombosis was followed by measurement of ^{125}I-fibrin deposits. The changes in radioactivity in the lungs were continuously monitored and recorded.

has also been used for the development of an acylated streptokinase-plasmin(ogen)-activator complex [32–34].

Animal experiments corroborated that, compared with the nonacylated complex, a smaller quantity of the acylated plasmin-streptokinase-activator complex (APSAC) was required to obtain thrombolysis [35].

The comparatively high thrombolytic effect of APSAC may be due to the fact that irreversible interactions with plasma inhibitors are blocked by acylation and that the acylated activator retains its affinity for fibrin. Peripheral activities of the fibrinolysis activator are largely excluded in order to prevent generalized hyperfibrinolysis and hemorrhagic complications resulting therefrom. The acylated complex presents another advantage: a single administration is sufficient to obtain thrombolysis, so that continuous infusion of streptokinase may be canceled. The *p*-aminobenzoyl- and *p*-anisolyl-plasmin(ogen)-streptokinase complexes have been developed and clinically tested as thrombolytic agents. The latter, named eminase, was introduced into clinical therapy [36].

3. Urokinase

Urokinase, the activator of fibrinolysis, excreted with the urine, has already been used as thrombolytic agent for some time. It is isolated either as low molecular weight urokinase (LMW-UK, MW 3,300 daltons) or high molecular weight urokinase (HMW-UK, MW 54,000 daltons). To prepare a urokinase delivery

system, we used *p*-amidinophenyl benzoate or *p*-nitrophenyl *p*-guanidinobenzoate [37,38].

The enzymatically inactive acyl-urokinase was reactivated in plasma at 37°C with a reactivation half-time of 8 minutes (benzoyl-urokinase) or 10 hours (*p*-guanidinobenzoyl-urokinase). Upon administration to rabbits, urokinase was more rapidly eliminated than either acyl enzyme (Fig. 11). The results suggest that urokinase is eliminated via the binding to plasma inhibitors. Thus, it could be shown that the clearance of urokinase is modified significantly on acylation.

For thrombolytic studies, benzoyl-urokinase was chosen. The thrombolytic effect of acylated urokinase as compared to the free enzyme was measured in vitro and in vivo. The in vitro experiments were carried out in an artificial circulating system composed of a ^{125}I-fibrin–labeled clot suspended in human plasma. The degree of thrombolysis was estimated from the released radioactivity (Fig. 12). From these experiments we concluded that the thrombolytic effect of benzoyl-urokinase is higher than that of urokinase.

The thrombolytic effect in vivo was studied in rabbits with a radioactive thrombus produced in the inferior vena cava. The radioactivity over the occluded venous segment was measured by a scintillation probe fitted with a collimator adapted to the anatomical conditions. Comparison of equivalent doses of urokinase and benzoyl-urokinase in the model of venous thrombosis showed that the acylated form of the enzyme was more active than the free enzyme [38].

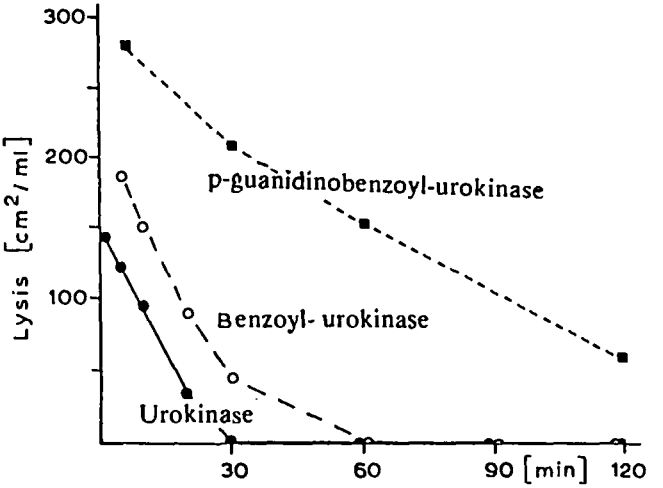

Figure 11 Fibrinolytic activity in rabbit plasma (estimated on fibrin plates) after i.v. injection of 50,000 IU/kg urokinase (U) or benzoyl-urokinase (BU) or guanidinobenzoyl-urokinase (GBU). (From Ref. 38.)

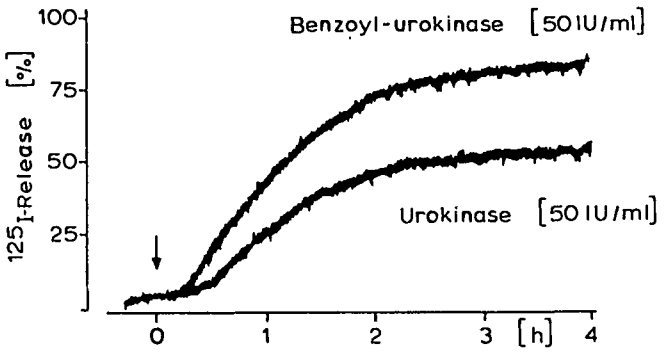

Figure 12 Lysis of radioactive human plasma clots (related to the increase in ^{125}I radioactivity) in a circulating plasma system by urokinase (U) and benzoyl-urokinase (BU) (50 IU/mL).

4. Tissue-Type Plasminogen Activator

Human tissue-type plasminogen activator (tPA) is currently available for therapeutic use, after it was successfully prepared from tissue culture or via recombinant DNA technique. Several investigators have reported an extremely short half-life both in experimental animals and in humans [39,40]. Therefore, we attempted to prepare stable acyl derivatives of tPA [41].

A series of acylated recombinant tPAs (r-tPA) was prepared by reaction of r-tPA with 4-amidinophenyl esters of substituted benzoic acids. The different acyl derivatives contained 5–10% of free enzyme. During deacylation, which is markedly influenced by pH and temperature, 80–100% of the amidolytic activity of the enzyme was recovered in active form.

For fibrinolytic studies the methoxybenzoyl derivative of r-tPA with a deacylation half-time of 10 minutes was chosen. Its fibrinolytic activity, which had been lost during acylation, was completely recovered by deacylation. This was demonstrated by the release of radioactivity from ^{125}I-labeled plasma clots. In these experiments r-tPA and the methoxybenzoyl derivative were found to possess the same fibrinolytic activity (Fig. 13).

C. Kallikrein

The plasma kinins kallidin and bradykinin are generated by the organic kallikrein released from glands and tissue or the serum kallikrein generated in blood from prekallikrein. Due to the vasodilator effect of plasma kinins, the kinin-generating

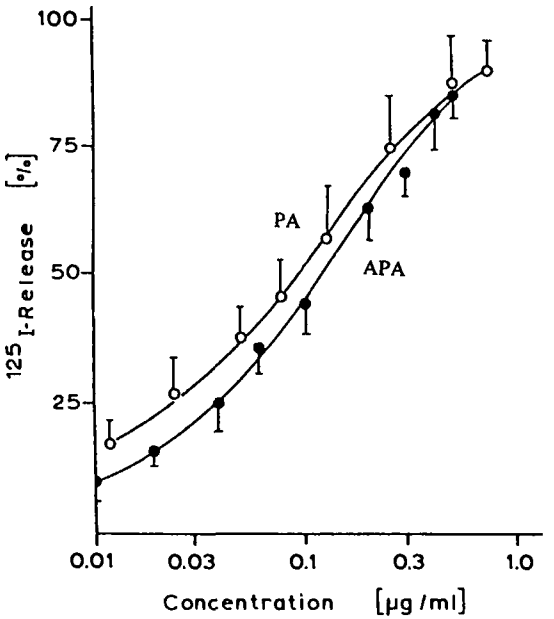

Figure 13 ^{125}I release from radiolabeled plasma clots. The clots were incubated with 1 mL human plasma and 50 mg r-tPA (PA) or acylated r-tPA (APA) at 37°C for 2 hr. (From Ref. 32.)

organic kallikrein has been used to induce peripheral vasodilation and decrease in blood pressure. However, in therapeutic use these drugs did not succeed because the enzyme administered as well as the kinin formed is relatively quickly inactivated in the circulation whereby the action becomes transient.

The acyl derivatives of kallikrein were prepared from porcine pancreatic kallikrein. In our experiments we used benzoyl-kallikrein. By deacylation the enzymatically active kallikrein was generated in plasma from benzoyl-kallikrein demonstrated by means of its amidolytic activity. From the time course of reactivation of benzoyl-kallikrein, a half-time of reactivation of 54 minutes was calculated. Benzoyl- kallikrein was protected from being inactivated by plasma inhibitors. Therefore, the kallikrein activity in plasma was higher after incubation with benzoyl-kallikrein than after incubation with the free enzyme. A comparatively high kinin activity was found in rabbit plasma. In human plasma, this effect was prevented by rapid degradation of kinin [42].

The effect of kallikrein and benzoyl kallikrein on kinin liberation in vivo was followed by a drop in blood pressure in rabbits (Fig. 14). Kallikrein administered

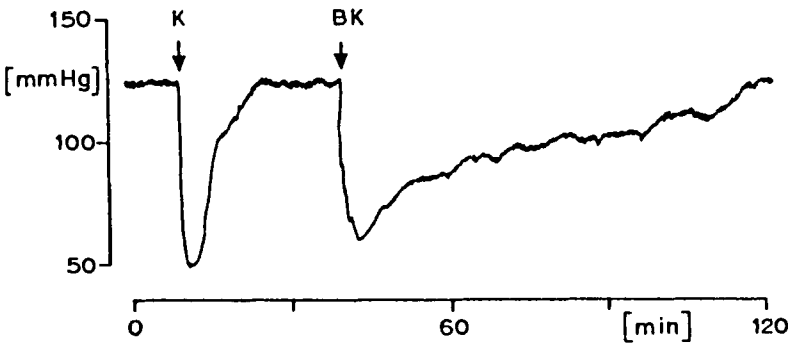

Figure 14 Effects of i.v. injection of 0.44 nmol/kg kallikrein (K) and benzoyl-kallikrein (BK) on mean blood pressure in rabbits. (From Ref. 43.)

intravenously causes a quick transient drop in systemic blood pressure. Besides sympathetic contraregulation and degradation of the pharmacologically active kinins, the inhibition of the administered kallikrein by natural plasma inhibitors may be of importance for the fast normalization of blood pressure [43]. Compared to the situation with the nonacylated enzyme, the duration of decrease in blood pressure after a bolus injection of acyl-kallikrein is longer and equals the time of reactivation. The time course of the decrease in blood pressure corresponds to that of an infusion of the same dose of kallikrein within 40 minutes. The results indicate that acylated kallikrein is protected from being inactivated by plasma inhibitors up to restoration of enzymatic activity.

IV. CONCLUSIONS

Guanidino and amidino phenylesters of substituted benzoic acids act as temporary inhibitors of serine proteases and may serve for the preparation of stable acyl enzymes acting as prodrugs [44]. The enzymes inactivated by active-site acylation regain their activity via deacylation in physiological environment. This opens up new pharmacological aspects to the acylated enzymes trypsin and related serine proteases and their action as proenzymes.

Experimental pharmacological investigations into selected acyl enzymes have shown that, besides the depot effect of the modified enzymes, their elimination characteristics changed as did, in some cases, their specific effect. In particular, as far as fibrinolytic enzymes are concerned, acylation could be shown to modify significantly the clearance of these enzymes. The prolonged circulation of

active-site–acylated fibrinolytic enzymes suggests that are least one significant physiological clearance mechanism, probably one requiring plasma inhibitors, is blocked by this modification.

Presumably, this concept of acylated enzymes may well be applied to further therapeutically interesting trypsinlike serine proteases. The pharmacological investigation into acylated enzymes has shown that, compared to the nonacylated enzymes, such preparations may well provide some advantages.

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5

Molecular Modeling of Pharmaceutically Important Targets

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I. INTRODUCTION

Knowledge about the three-dimensional (3D) structure of an enzyme is crucial for the understanding of its function, and can be of great importance for the design of drugs or the tailoring of these enzymes into pharmaceutically useful therapeutics. The benefits of the availability of three-dimensional coordinates are easiest seen for lead optimization. If a lead compound (e.g., an enzyme inhibitor) is available, one can determine the structure of the complex of the enzyme with the pharmacophore. A study of this complex will rapidly lead to suggestions about new and perhaps even better compounds. In the absence of a lead compound, the structure of a protein can be used to search for cavities or concave surface areas. Potentials for atomic probe groups can be calculated [1], and it can be attempted to fill the target-binding site with appropriate atoms organized in such a way that they overlap best with the potentials. At the same time it should still be possible to synthesize a real molecule that contains these atoms in the required relative orientation [2].

Knowledge of the three-dimensional structure of pathogenic proteins (e.g., viruses) is important in the design of peptide vaccines. Rather than trying all possible peptides, one only needs to try those peptides that are predominantly at the surface in the pathogenic protein. Understanding nonlinear epitopes is

virtually impossible without knowledge of the structure of the pathogenic protein [3].

Monoclonal antibody techniques allow us to design antibodies targeted at a specific pathogen. There is no need saying that a thorough understanding of the structures of the antibody and the pathogen is required. The humanization of antibodies—needed to avoid side effects when used therapeutically—requires crafting the hypervariable loops of one antibody (e.g., from mouse or goat) on the scaffold of another (human) antibody [4,5]. This crafting or transplanting of loops needs to be done with great precision because misplacing one single atom by as little as 1.0 Å could completely destroy the binding. Thus, a thorough understanding of both the interactions between the hypervariable loops and of the interactions between these loops and the scaffold is required [6–11].

In many cases the most important aspect of structural studies is that knowledge of the structure of a protein leads to improved understanding of its function. For instance, if a protein has to be administered for lifetime or long-term replacement therapy (e.g., insulin, growth hormone, factor IX), it is important to know all about its structure-function-stability relationships. A longer turnover time or a higher specific activity, for example, allows for a smaller dose to be administered, which has medicinal and financial benefits.

X-ray crystallography, NMR spectroscopy, and molecular modeling are the three techniques to obtain structural information. X-ray crystallography can provide very precise atomic information. However, crystallography is a technically and computationally elaborate technique. NMR spectroscopy has as its main advantages that no crystals are needed and that dynamic information about the protein of interest can be obtained. The main disadvantages of NMR are that the atomic positions cannot be determined with the same precision as by x-ray crystallography, and that often large amounts of protein are required.

In the absence of experimental data, model building on the basis of the known three-dimensional structure of a homologous protein is at present the only reliable (computational) method of obtaining structural information [12]. Comparisons of the tertiary structures of homologous proteins have shown that three-dimensional structures have been better conserved in evolution than primary structures [13–17], supporting feasibility of model building by homology. Differences between three-dimensional structures increase with decreasing sequence identity and accordingly the accuracy of models built by homology decreases [18,19]. The errors in a model built on the basis of a structure with 90% sequence identity may be as low as the errors in crystallographically determined structures, except for a few individual side chains [19–20]. If, as a test case, a known structure is built from another known structure, then in the case of about 50% sequence identity the RMS error in the modeled coordinates can be as large as 1.5 Å, with considerably larger local errors. If the sequence identity is only

around 25%, the alignment is the main bottleneck for model building by homology and large errors are often generated. With less than 25% sequence identity, the homology often remains undetected. In the absence of a structure with sufficient homology to the protein of interest, secondary structure prediction can provide important information, but not sufficient three-dimensional detail to allow, for example, for rational drug design. Figure 1 shows the key limiting factors in modeling as a function of the percentage sequence identity.

The deviations between a model built by homology and the "real" x-ray structure vary throughout the molecule, the largest deviations occurring in loops at the protein surface. In regular secondary structure elements (helix, strand) and in the hydrophobic core, models normally are more accurate.

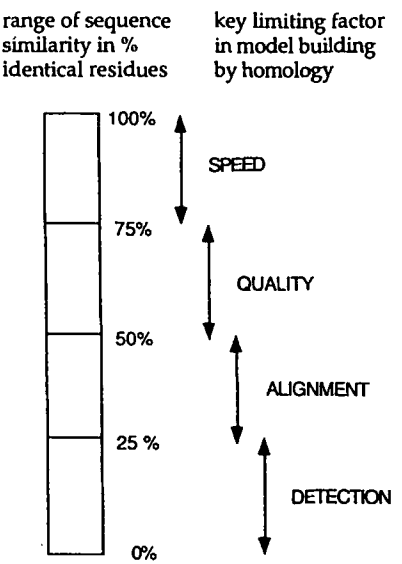

Figure 1 The main limiting steps for model building by homology as function of the percentage sequence identity between the structure and the model. *In case of high homology*: Improvement of the way side chains are positioned in the modeled structure and improvement of the database techniques needed to do this fast and accurate. *In case of intermediate homology*: Improvement of loop modeling and development of techniques to check the quality of models. *In case of low homology*: Improvement of the sequence alignment that is at the basis of the model-building procedure and evaluation of the significance of alignments. *In case of no detectable homology*: Development of secondary structure prediction and ab initio 3D modeling techniques and incorporation of chemical information in all modeling procedures.

At present, most protocols for model building by homology start from the assumption that, except for insertions and deletions, the backbone of the model is identical to the backbone of the known structure. In practice, domain motions and "bending" of parts of molecules with respect to each other is often seen. However, even in case of significant bending, short-range interactions will not differ very much and the model will be perfectly adequate for rational drug design or for analysis of an active site.

In this chapter several aspects of 3D model building by homology will be described. It will become clear that sequence alignments often are the limiting step for the quality of the homology modeling and an evaluation of the minimal required homology between the sequence and the structure that will serve as the modeling template is very important. At present, few published examples of successful modeling of pharmaceutical enzymes exist, and when such examples are presented, the main aim normally is the design of inhibitors. Three examples are selected to illustrate some of the typical aspects of molecular modeling of proteins in general, and of enzymes in particular.

II. MODEL BUILDING BY HOMOLOGY

A. High Homology: Placing New Side Chains in the Structure

In case of high sequence homology between the template structure and the model, the main problem lies in the mere replacement of a few side chains. Given that the backbone remains constant, the problem is reduced to finding the correct side chain conformation (the correct rotamer). Often this can be done by hand [21], but if done in an automatic fashion, two main strategies can be followed.

Residue side chains can be "grown" during molecular dynamics runs [22–27]. This is a very time-consuming operation, but it can be beneficial if mutations in an active site have to be correlated with differences in binding constants of drugs. Knowledge of the cause of differences in energetics of the binding of existing drugs might influence the decision to synthesize a new drug or not.

Alternatively, one tries to extrapolate the correct rotamer from examples of residues in a similar environment in the database of known structures. The main problem here is how to define similar environments. In practice, similar environments for residues have most often been defined as having the same secondary structure (α-helix, β-strand, or loop). Until recently, side chains were normally placed using standard rotamer libraries [28–30]. A variety of procedures have been used to get rid of van der Waals clashes. These range from manual

adaptation [21] to complicated schemes involving energy calculations [31] or Monte Carlo procedures combined with energy calculations [32,33].

The latest developments in modeling are position-specific rotamer searches (Fig. 2). Dunbrack et al. and Vriend et al. showed that the preferred rotamer is to a large extent determined by a local backbone [34,35]. Database searches for peptides with the same local backbone and the same amino acid type in the middle often reveal a more restricted pattern of side-chain conformations than one would expect from generalized rotamer libraries. Position-specific rotamer distributions are obtained by searching the database for segments of residues that fulfill two criteria:

The segment has a local backbone conformation similar to that of the fragment that has to be modeled.

The segment has the same residue type at the middle position.

Additional constraints can be placed on the accessibility of the middle residue and on the types of residues next to the central one (e.g., hydrophobicity, size, etc.). Typically these segments are five or seven residues long and the RMS difference between superposed alpha carbon atoms is kept at maximally 0.5 Å. Levitt and De Filippis et al. recently described such methods [36,37].

The idea behind using such searches is to extract from the database of known protein structures examples of local structures that look as much as possible like the local structure to be modeled. The more the database fragment looks like the

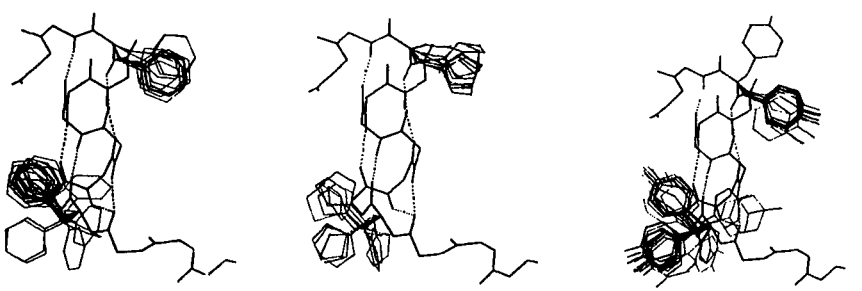

Figure 2 Position-specific rotamer distributions for Phe, His, and Tyr at the second and penultimate position in a short α-helix. It can be seen that only one rotamer is allowed at the C-terminal end, whereas the rotamer distribution is residue dependent at the N-terminal end of the helix. These rotamer distributions are obtained by searching the protein structure database for segments of residues that fulfill two criteria: (1) the segment has a local backbone conformation similar to that of the fragment around the residue that has to be modeled, and (2) the database segment has the residue type at the middle position that has to be modeled.

fragment to be modeled, the more likely it is that the modeling will go well. Position-specific rotamer searches extract from the database as much local information as possible given the size of the present-day protein structure database. These searches are thus an important tool in modeling. In fact, it is our experience that using position-specific construction of side chains gives the best model building by homology results (smallest all-atom RMS deviations) obtainable to date when one known protein structure is modeled from another.

De Filippis et al. [37] compared the structures of 84 mutants with the corresponding wild-type structures. Their main conclusions are that point mutations seldomly lead to large alterations in the overall structure of the protein, and that it is easy to predict the structure of a point mutant. They also determined a set of simple rules that can be used to predict the structure of a mutated residue given the structure of the wild-type protein. These rules are described in Figure 3. It is to be expected—although it is not yet proven—that this simple scheme can be extrapolated to model building by homology in cases of high homology.

B. Intermediate Homology 1: Building Loops If There Is an Insertion in the Model

In case of more than 75% sequence identity between the template structure and the model, one seldom encounters insertions or deletions, and when they appear they normally are short.

The insertion of loops is one of the main problems in model building with intermediate homology. If an insertion in the sequence occurs relative to the structure, there is no template to model on, and other techniques have to be applied. The main techniques used to model loops are:

Searching databases for loops with endpoints that match the residues in the structure between which the loop is to be inserted. This technique is based on the same distance geometry loop searching algorithm as described above for the retrieval of position-specific rotamers from the structure database [38]. Many molecular graphics and modeling programs use this technique (e.g., WHAT IF [39], QUANTA, Hydra [40], FRODO [41], Insight [42], O [43], BRAGI [44]).

Ab initio building can be done in many ways, but a few points are common to all techniques: the fact that endpoints have to match is a very strong constraint. Furthermore, Van der Waals clashes have to be prevented, and all rules about proper stereochemistry and energetics should be obeyed. Many articles have been published about these topics [45–51].

Molecular class-specific rule-based modeling. A good example is the modeling of hypervariable loops of antibodies. Tramontano et al. realized that five of the six hypervariable loops choose their conformation from a limited

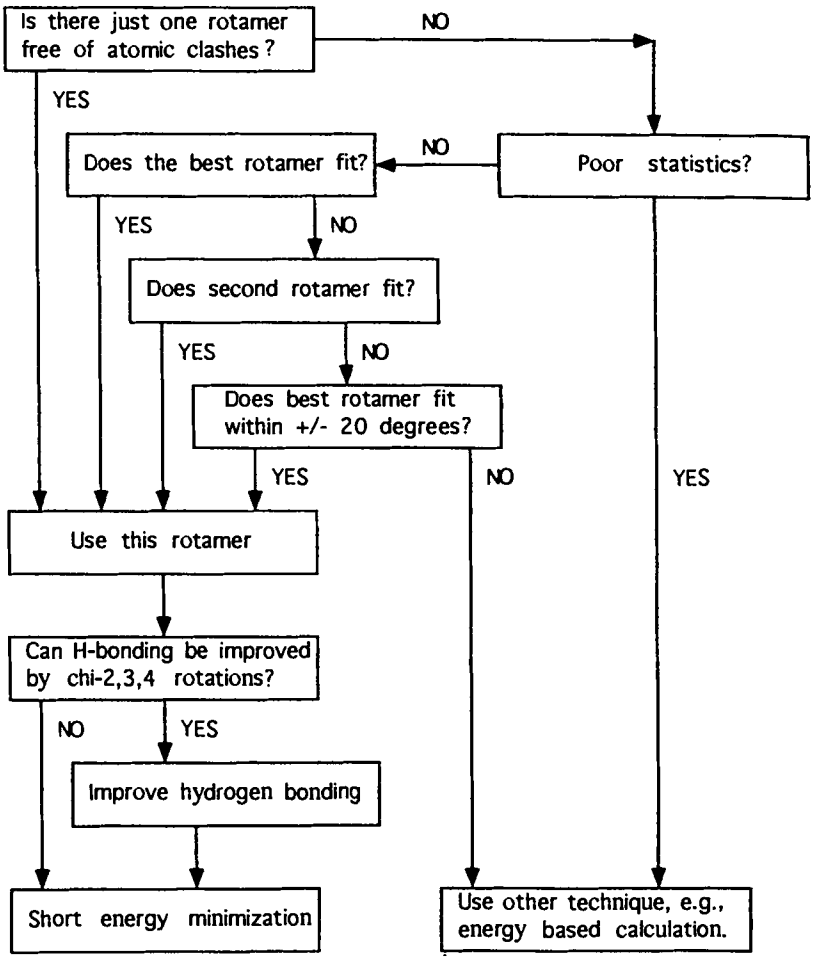

Figure 3 Decision scheme for the prediction of the conformation of a mutated residue. This scheme is based on two observations: (1) A point mutation seldom leads to large alterations in the overall structure of the protein. The mutated residue adapts to the structure of the rest of the protein rather than the other way around. (2) Most residues sit in the statistically preferred conformation, and when exceptions occur, they can normally be explained on the basis of hydrogen-bonding patterns. (Adapted from Ref. 37.)

repertoire [10,52]. They also observed that there is a correlation between the loop type and the presence of some key residues. Conversion of this knowledge into a computer program led to results that were astonishingly good. In one case the predicted structure was more correct than the initial x-ray structure.

C. Intermediate Homology 2: Verification of the Quality of the Model(s)

All models built by homology will have errors. Side chains can be placed incorrectly, or whole loops can be misplaced. As with most errors, they become less of a problem when they can be localized. For example, looking at an enzyme it is usually not important that a loop far away from the active site be modeled incorrectly. The most important step in the process of model building by homology is therefore undoubtedly the verification of the model, and the estimation of the likelihood and magnitude of errors.

There are two principally different ways to estimate errors in a structure:

Determination of the energy of the structure using energy minimization and molecular dynamics programs.

Determination of normality indices that indicate how well a given characteristic of the model resembles the same characteristic in real structures.

There is continuous progress in molecular dynamics and energy minimization. The main topics are force field improvement [26,53–56], incorporation of effective solvation terms [57], ab initio modeling of small proteins [49–51,58,59], the incorporation of real data for x-ray refinement [60] and NMR structure determination [61]. However, much work still has to be done to improve the force fields used for energy calculations before any but trivial errors can be detected this way.

Normality indices for structures, on the other hand, already have proven their power in structure verification. Many characteristics of protein structures lend themselves to normality analysis. Most of them are directly or indirectly based on the analysis of contacts, either interresidue contacts or contacts with water. Some published examples are:

General checks for the normality of torsion angles, bond angles, bond lengths, etc. [62–64] are good controls for the quality of experimentally determined structures, but are less suitable for the evaluation of models because the better model-building programs simply do not make these kinds of errors.

Inside/Outside distributions of polar and apolar residues can be used to detect completely misfolded models [65].

Solvation potentials can detect local errors as well as complete misfolds [64].

Residue transfer energy is another way of looking at inside/outside distributions [66].

Packing rules have been implemented for structure evaluation [67].

Atomic contacts that are not abundant in the protein structure database are good indicators of local model-building problems [63]. Atomic contacts are observed because they are energetically favored. Real structures cannot tolerate too many unfavorable interactions. Thus for a model to be correct, only a few infrequently observed atomic contacts are allowed. This "quality control" of local packing has proven to be the most powerful tool for the detection of abnormal structures.

Both techniques for the verification of protein structures described above (energy calculations and statistical determination of normality) can of course also be applied to the prediction of drugs and to the evaluation of predicted protein-drug interactions.

D. Low Homology 1: Database Size and the Significance of Alignments

Many aspects of modeling are limited by the size of the database in which one performs searches or from which one derives structural preferences.

As described above, the transfer of structural information to a potentially homologous protein is straightforward if the sequence similarity is high, but the assessment of the structural significance of sequence similarity can be difficult when sequence similarity is low or restricted to a short region. This indicates one of the key problems: the shorter the length of the alignment, the higher the level of similarity required for structural significance.

Sander and Schneider performed an empirical determination of homology thresholds by studying thousands of sequence alignments within the PDB database [19]. One has to keep in mind that perfect sequence similarity not always implies perfect structural agreement: a protein crystal structure may vary, typically in loop regions or in domain orientation, as a result of different substrate or cofactor interaction, complex formation or crystal contacts. The curve in Figure 4, however, provides a reliable rule of thumb for those who wonder if their enzyme can be modeled with any degree of confidence.

E. Low Homology 2: Using the Structure to Improve the Alignment

If the percentage sequence identity between the structure and the sequence to be modeled is well below 50%, then the quality of the sequence alignment is the

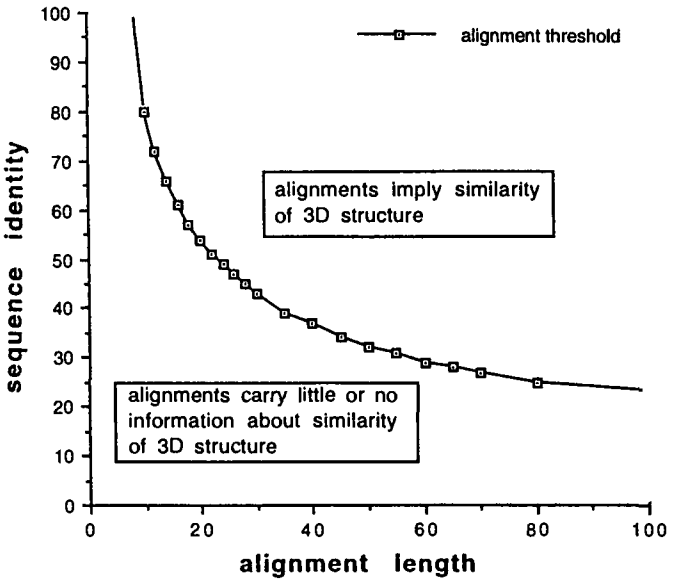

Figure 4 Homology threshold for structurally reliable alignments as a function of alignment length (number of aligned amino acids). The homology threshold (curved line) divides the graph into a region of safe structural homology where essentially all fragment pairs have good structural similarity and a region of dubious homology where some fragment pairs are structurally similar but most are not.

limiting factor for the quality of the overall model. Most sequence-alignment programs only use sequence information. However, in case of model building by homology, more information is available in the form of a structure. Use of this information in principle must lead to an improved alignment.

Several authors have described techniques to align two proteins in cases where the structure of one of the proteins is known [68–82]. Rather than using the sequence of the known structure as input to the alignment procedure, a structure-dependent sequence profile is set up and the sequence of the unknown structure is aligned with this profile. The basic idea behind these structure dependent profiles is best explained by an example:

Sequence alignment programs use the dynamic programming algorithm to quickly find the best of all possible alignments between two sequences. For each alignment a score is determined. This score is a function of all pairs of matched residues. For example, a positive score is given if two matched residues are identical. This score is higher for critical residues like cysteine, proline, or

tryptophan. The score is about neutral in case similar residues are matched, e.g., serine with threonine or glutamic acid with aspartic acid. A negative score is given if the physicochemical characteristics of the matched residues are very different, like, for example, a match between the small hydrophobic valine and the large and polar glutamic acid. This scoring scheme has one clear problem: all identical matches between two residues always get the same score. Every serine-to-arginine mutation always receives the same score. A serine at the surface of the protein, however, can often easily be replaced by an arginine, but a serine in the core of a protein is very unlikely to be replaced by an arginine. The aforementioned profile techniques try to incorporate this structure-dependent information to arrive at better sequence alignments.

A second goal of these new methods is the detection of structural homologies in the "twilight zone" of sequence similarity (less than 25% identity), e.g., the structural similarity between carboxypeptidases A and N (sequence identity 18%). None of the sequence-structure–alignment methods has been proven to consistently and generally detect remote structural homologies with a high score and with the correct alignment. Nevertheless, several interesting examples have been reported, e.g., the relation between actin and heat-shock protein hsp70 [74] or between phycocyanin and myoglobin [76].

F. No Detectable Homology: Prediction of Protein Secondary Structure or Protein Function

If building by homology of a three-dimensional model is not feasible because no structure with significant sequence homology can be found, one would like to be able to get at least an impression about the secondary structure or the function of the protein of interest. Because the sequence of a protein determines its structure, it must in principle be possible to reconstruct the secondary and tertiary structure from the sequence information alone. Over the last 20 years many programs have been written for this purpose; the one most widely known undoubtedly being the Chou and Fasman method [83]. In practice, however, secondary structure prediction methods have long been unable to produce higher precision than 55–64% on a three-state (α-helix, β-strand and loop) model [83–90]. Keeping in mind that a program that always predicts all residues to be helical will score 35%, one realizes that 55% correct (that means 45% wrong!) is not enough to draw conclusions about the structure. Rost and Sander have recently achieved a substantial increase in both the accuracy and quality of secondary structure predictions using a neural network algorithm [91]. The main improvements come from the use of multiple sequence alignments (better overall accuracy), from "balanced training" (better prediction of β-strands), and from "structure context training" (better prediction of helix and strand lengths). Their method achieves a three-state

prediction accuracy of 71.4%, significantly better than any previous method. The improved distribution and accuracy of helices and strands makes the predictions suitable for use in practice as a first estimate of structural type of newly sequenced proteins.

Many classes of enzymes have their own specific sequence fingerprint. Often a sequence is determined that shows no significant homology to any other sequence, except for a very small pattern of residues. Using a library of function-specific patterns one can in many cases still determine the functional class of the protein, despite the fact that no structural information is available. The Prosite [92] sequence pattern database is the most extensive depository of function-determining sequence patterns.

G. No Detectable Homology: Using Chemical Information to Build a Model

A novel development in the molecular modeling field is the incorporation of chemical information. If, for example, it is known that two residues are part of the active site of an enzyme, then these residues should be modeled spatially close enough to participate in the enzyme mechanism. Other chemical information that can be used to put constraints on the model are the knowledge that residues lie at the surface (from proteolysis studies or antibody binding studies), the existence of cysteine bridges, or the relative orientation of aromatic rings (from fluorescence measurements). Of course, chemical information has so far always been used in modeling, but only in two ways: to manually interfere in the sequence alignment and to check the final model. The incorporation of chemical information in automated model-building procedures is still in its infancy, but some of the examples shown below make clear that this technique has the potential to become a must for every serious modeling study.

Frigerio et al. [93] showed how mutagenesis studies can be used to solve ambiguities in the model. In their case, point mutations were designed to be useful in the modeling process. In other cases large amounts of mutagenesis experiments already exist prior to the modeling. A good example of the usage of biochemical, biophysical information, and the results of mutagenesis work is provided by G protein–coupled receptors. The second example below is devoted to this work.

III. EXAMPLES

The next sections will present three examples of modeling studies. These studies have been published elsewhere, but are given here with a different emphasis.

Rather than concentrating on the results and conclusions, the modeling aspects will be described extensively. The first two examples show how novel techniques and the incorporation of chemical data solved problems where classical homology modeling would have failed. The second example, G protein–coupled receptors (GPCRs), strictly speaking does not deal with pharmaceutical enzymes. They provide however, the best documented example of model building without homology. Actually, several of these receptors show proteolytic activity; some cleave their substrate, and some cleave themselves upon substrate binding. Thus, although it will be a long time before receptor models will be used for anything but drug design, at least a couple of them are true enzymes. The third example was chosen because it shows that modeling is not always difficult and that very simple methods can often be used to build models that allow a deepening of our understanding of the structure and function of the molecule.

A. Example 1: Modeling the Active Site of Carboxypeptidase N

Basic carboxypeptidases have recently received renewed interest in medical science, because it is evident that these enzymes are involved in peptide hormone maturation. Many peptide hormones are synthesized as precursor proteins that are often cleaved at pairs of basic amino acids such as Arg-Arg or Lys-Arg. Basic carboxypeptidases can then generate the correct product by removal of the C-terminal basic residue [94].

The human plasma metallo-protease carboxypeptidase N (CPN, arginine carboxypeptidase, anaphylatoxin inactivator, kininase I, EC 3.4.17.3) catalyzes the release of the basic amino acids lysine and arginine from the C-termini of peptides and proteins such as bradykinin and kallidin [95], the anaphylatoxins C3a, C4a, and C5a [96,97], fibrinopeptides 6A and 6D [98], hexapeptide enkephalins [99], protamine [100], and the creatine kinase MM-isoenzyme [101,102]. Its most likely physiological function is to protect the organism from the actions of potent peptides, which may escape from tissues or be released in the circulation.

Carboxypeptidase N is synthesized in the liver [103] and circulates as a 280,000 M_r tetrameric complex. It is composed of two identical high molecular weight subunits (M_r 83,000), which are heavily glycosylated and lack enzymatic activity, and two identical low molecular weight subunits (M_r 55,000), which contain the active center [104,105].

Hendriks et al. [106] modeled the structure of the enzymatically active subunit of human plasma carboxypeptidase N based on the structure of bovine pancreatic carboxypeptidase A. The aim of the study was twofold: improved understanding of the way the molecule functions and understanding the

molecular basis for the differences in substrate specificity between the carboxypeptidases.

1. Sequence Alignment

The sequence alignment between the different carboxypeptidases was the largest problem encountered upon modeling CPN. In pairwise alignments percentage sequence identities as low as 15% were observed. Therefore, the sequences were aligned using combined information from several approaches. The methods applied were intended to delineate reliable information about location and extent of sequence similarity. A sequence-comparison method based on amino acid properties [107,108] was used to calculate dotplots between carboxypeptidase N and the other sequences. Given the low overall similarity in this family of proteins, the sequences had to be compared as a set. This was done using an approach based on the consistent occurrence of similarities in the entire set of pairwise comparisons [109,110]. This method not only calculates a multiple sequence alignment but at the same time highlights those parts of the alignment that can be trusted more than others, and hence can be modeled with more confidence. The final sequence alignment is shown in Figure 5. The sequence-alignment techniques used allow us to determine those regions where the results from model building are more likely to be correct. Fortunately, these regions include almost all residues around the active site. Sixteen out of the 37 (43%) residues within 8 Å of the Gly-Lys dipeptide docked into the active site of CPN are identical in comparison with carboxypeptidase A, which gave great confidence about the quality of the model around the active site (Fig. 6).

2. Model Building

The atomic model of the active subunit of CPN was built using the structure of CPA [111] and replacing its sequence by the CPN sequence [112] following the protocol described by Vriend and Eijsink [113] and De Filippis et al. [37] using the homology modeling module of the program WHAT IF [39]. The subsequent steps in this modeling procedure are summarized in Table 1.

The model was built and optimized in the absence of inhibitors. Dipeptide inhibitors were docked in the energy-minimized model. The coordinates of the slowly hydrolyzed substrate glycyl-L-tyrosine [114] for CPA were used to guide the (manual) docking. Atomic coordinates were obtained from the Protein Data Bank [115]. Energy minimization and molecular dynamics were carried out with GROMOS [116].

Because of the low homology and the presence of a 12-amino-acid insertion (CPN 208–219), two external loops (CPN 146–169 and 208–219) were not built.

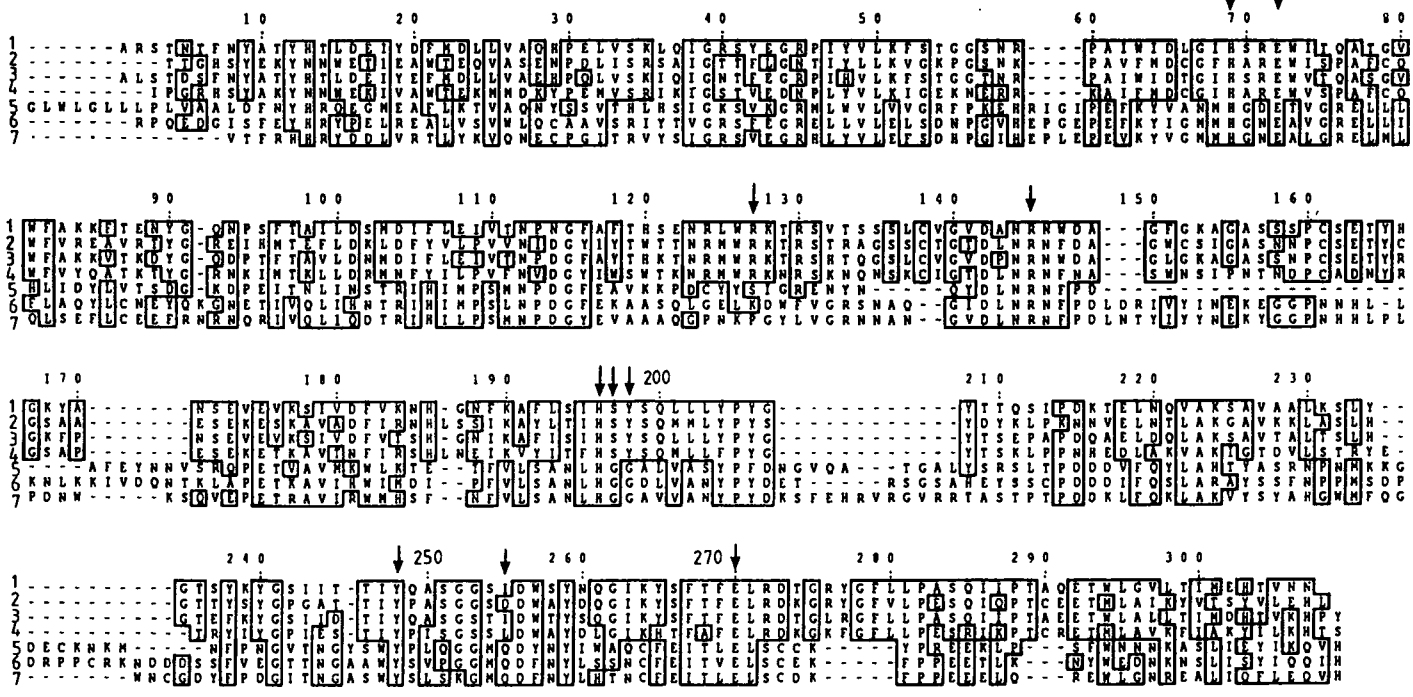

Figure 5 Multiple sequence alignment of carboxypeptidases. Similarity of the enzymatically active subunit of human carboxypeptidase N (7) to carboxypeptidase A from bovine (1), rat (3), human mast cell (4), bovine carboxypeptidase B (2), human carboxypeptidase M (5), and bovine carboxypeptidase H (6). Residues identical and conservative changes in at least four proteins are boxed. Arrows indicate active site residues. (From Refs. 119–123.)

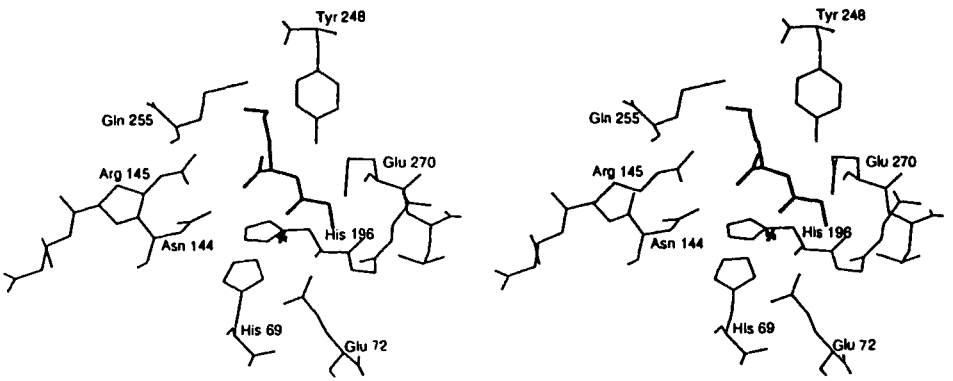

Figure 6 Amino acids in the active site of carboxypeptidase N. The dipeptide glycyl-L-tyrosine is docked into the active site. The active site zinc is coordinated to His 69, Glu 72, and His 196. Arg 145, Asn 144, and Tyr 248 provide specificity for substrates bearing a free terminal carboxylate. Gln 255 forms a hydrogen bond with the side chain of the carboxy-terminal arginine or lysine. Glu 270 promotes the nucleophilic attack of a water molecule at the scissile peptide bond.

These loops, however, are situated far from the active center and are unlikely to interfere with substrate binding or enzymatic activity.

3. Specificity

Ile-255 is a crucial residue in the hydrophobic pocket of CPA and gives CPA its preference for substrates with aromatic and branched-chain C-terminal amino

Table 1 Flowchart of the CPN Modeling Procedure

Sequence alignment (difficult because of low homology)
Verification that the residues known from experiments to be functionally equivalent (active site residues) line up in the alignment
Exchange of the side chains of residues in CPA structure by CPN residues that according to sequence alignment belong at this position
Verification that model is in agreement with all rules known about normal protein structures
Correction of the sequence alignment based on the evaluation of the model
Iteration of the last three steps
Manual correction of some residues where the modeling was too complicated for automatic procedures
Optimization of the model structure using energy minimization

acids [117]. CPN has a glutamine at this position that has its side chain pointing in the same direction. This Gln forms a hydrogen bond with the side chain of the carboxy-terminal Arg or Lys of the substrate. In carboxypeptidase B (CPB), there is an Asp at this position. This negatively charged residue will attract the positively charged Arg or Lys better than the Gln in CPN. This probably explains the lower K_m on the substrates hippuryl-L-arginine and hippuryl-L-lysine in comparison with CPN [106,118]. [CPB: K_m is 0.2 mM (Hip-Arg) and 0.015 mM (Hip-Lys), k_{cat} is 6300 min^{-1} (Hip-Arg) and 5200 min^{-1} (Hip-Lys); CPN: K_m is 1.8 mM (Hip-Arg) and 4 mM (Hip-Lys), k_{cat} is 780 min^{-1} (Hip-Arg) and 4200 min^{-1} (Hip-Lys).]

To calculate the substrate specificity of CPN, the GRID potential energy method [1] was used to determine energetically favorable binding sites (Fig. 7). This led to several suggestions for substrate specificity and possible peptide inhibitors. New dipeptide substrates were synthesized, and the results confirmed the predictions made from the calculations [106].

This example shows that modeling by homology, when only 18% sequence identity to a known structure exists, can lead to a model sufficiently reliable to understand the molecular basis for the substrate specificity and to qualitatively predict substrates or inhibitors. The experimental confirmation of the correctness of the model around the active site gives confidence that future experiments, which will be based on this structure, have a good chance of being successful.

B. Example 2: Modeling G Protein–Coupled Receptors

G protein–coupled receptors constitute a large group of membrane-spanning receptors of diverse biological function. It is estimated that at least several hundred different members of these proteins exist [124–128]. The basic function for all GPCRs seems to be similar. An external signal is received; this is normally a ligand but in the case of opsins, a photon alters the structure of the bound retinal. This external signal is transduced through the membrane to the cytosolic side where a G protein is activated. The signal reception and transduction is basically performed by the membrane embedded part of the receptors. The specificity for G proteins is most likely determined by the cytosolic loops [129]. The receptors have been divided into three superfamilies [130]:

1. Rhodopsinlike receptors
2. Secretinlike receptors
3. Metabotropic glutamate receptors

Table 2 lists some of these receptors and their natural agonists.

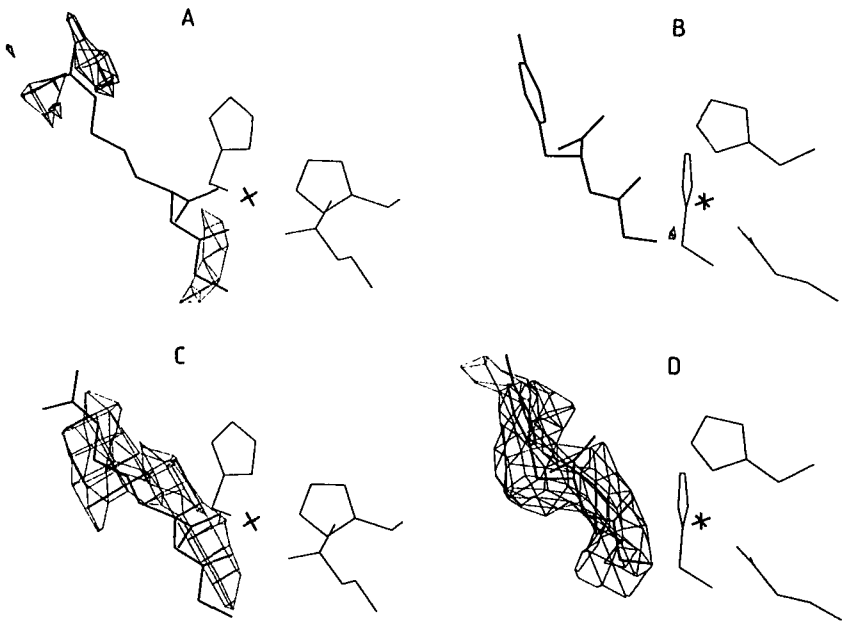

Figure 7 Contour plots of the energetically favorable binding sites in the substrate binding cleft of carboxypeptidase N and A. Contours are plotted for a sp3 positively charged amino group for CPN at −15 kcal/mol (A) and for CPA at −15 kcal/mol (B), for a methylene group for CPN at −3 kcal/mol (C) and for an aromatic carbon atom for CPA at −2 kcal/mol (D). In A and C the dipeptide Gly-Arg and in B and D the inhibitor Gly-Tyr are shown with thick solid lines. The three Zn-binding ligands (His 69, Glu 72, His 196) as well as the active site Zn are shown. Using the $-NH_3+$ probe group and $=NH_2+$ probe group, as present in lysine and arginine respectively, we found a bifurcated energetically favorable pocket in the active site cleft of CPN in a position that coincides with the place were the guanidine group of the carboxy-terminal arginine or the $N\zeta$ of a lysine would fit (A). This pocket is not observed in carboxypeptidase A (B). Using an aromatic carbon as probe group, carboxypeptidase A shows a wide energetically favorable pocket where an aromatic ring can fit (D), whereas in carboxypeptidase N a narrow pocket is available that is not wide enough to accommodate a phenyl ring (result not shown).

No direct three-dimensional structure determination of any GPCR has been carried out yet. However, the structure of the membrane-embedded helices of bacteriorhodopsin has been solved by electron crystallography [131]. Experimental data from deletion mutations, antibody targeting, and proteolytic digestion experiments provide evidence that the overall features of GPCRs and

Table 2 Some G Protein-Coupled Receptors and Their Endogenous Ligands

Receptor	Agonists
Adrenoceptors	Adrenaline, noradrenaline
Serotonin receptors	Serotonin
Dopamine receptors	Dopamine
Opsins	Activated by light
Calcitonin receptors	Calcitonin
Growth hormone receptors	Growth hormone
Bradykinin receptors	Bradykinin, des-Arg bradykinin
Opioid receptors	Opioids
Tachykinin receptors	Substance P/Substance K
Thrombin receptor	Thrombin

bacteriorhodopsin must be similar. Bacteriorhodopsin has seven transmembrane helices organized in a kidney-shaped manner with the nearest neighbor helices running antiparallel.

It has been determined experimentally that GPCRs also possess seven transmembrane helices (for reviews, see Refs. 132,133). Many attempts have been made to derive three-dimensional models of GPCRs based on this similarity with bacteriorhodopsin [134–136]. This modeling has shown that point mutations and inhibitor binding can be in agreement with the assumption that the seven helices of GPCRs and bacteriorhodopsin are similar. A recently published low-resolution two-dimensional projection map of bovine opsin [137], obtained with electron diffraction, is supportive of these ideas.

Model building has to start with a sequence alignment between the GPCRs and bacteriorhodopsin. A problem is that standard sequence-alignment techniques use standard amino acid exchange matrices. These matrices have been optimized for the alignment of water-soluble proteins and are far from ideal for membrane-embedded proteins. Another problem is that the overall sequence identity between GPCRs and bacteriorhodopsin is far too low to allow for model building by homology by standard techniques [19,39].

A large amount of information (sequences, agonist and antagonist binding, mutation studies, etc.) is available for the rhodopsin-like G protein–coupled receptors. However, only limited information exists about the secretin-like and metabotropic glutamate receptors, and too few sequences have been determined yet to define sequence-conservation patterns in these subfamilies. Consequently,

most modeling studies so far have concentrated on the rhodopsinlike G protein–coupled receptors.

The work by Cronet et al. [136] made clear that general rules for residue exchanges in membrane helices are hard to derive because residue preferences are very position specific. A good Dayhoff-type matrix can therefore not be derived for the transmembrane helices, which makes it difficult to use standard sequence-alignment techniques.

An initial alignment of the sequences of about 100 known GPCRs, extracted from the SwissProt [138] and PIR [139] databases, was made by inspection, using knowledge about mutations that cause similar effects in different receptors. From this multiple sequence alignment a profile was derived. The correspondence of this profile to the bacteriorhodopsin structure was based on the combined chemical knowledge of all aligned sequences. For example, all residues in all sequences known to be involved in ligand binding had to point inwards in the final models. In this profile the very large insertions that often occur between helices V and VI were left out. The profile obtained from the initial manual alignment was used to realign all sequences. The process of rebuilding the profile from the multisequence alignment and aligning all sequences one by one against the profile was iterated until no changes occurred between two subsequent steps [140].

After aligning all GPCR sequences, it was possible to extrapolate the massive body of chemical information available for these molecules. The basic assumption is that if, for example, it has been measured that a residue is facing the lipid in one of the receptors, all equivalent residues in all other receptors are facing the lipid too. The sequence alignment of the helices of the GPCRs has been used to build models of the helical parts of 250 GPCRs. These models have been deposited and are awaiting verification or falsification either by comparison with chemical data or by solving the structure of one of them. Until that time they are like all models: most likely not totally correct, but very useful to aid with the study of these molecules.

C. Example 3: Modelling the Lipase of *Rhizopus javanicus*

Triacylglycerol lipases (EC 3.1.1.3) are attracting renewed attention since they were demonstrated to be active in organic solvents and to be suitable catalysts in industrial important reactions, such as the synthesis of flavors, emulsifiers, and chiral compounds, and the transesterification of low-value fats to triacylglycerols of high commercial value. Fungi are a particularly valuable source of lipases because the enzymes produced by the majority of them are extracellular and readily separable from the mycelia after fermentation. The recent availability of

these enzymes on a large scale is leading to the development of new techniques in fields as diverse as food processing, detergent manufacturing, fine chemical synthesis, and sewage treatment and is also generating interest in the molecular engineering of these lipases toward forms that can be used in harsher conditions (such as low pH) or that possess altered substrate specificities.

The three-dimensional structures of human pancreatic lipase and *Rhizomucor miehei* lipase have recently been elucidated [141–143]. Among the lipases purified from different sources—mammals, bacteria, fungi, and plants—the fungal lipases from *Rhizopus* species exhibit a remarkably broad pH zone of stability and activity, extending from pH 3 to 9.

Uyttenbroeck et al. [144] described the purification and characterization of the acid-resistant lipase from *Rhizopus javanicus*. The sequences of the lipases from *Rhizopus javanicus* and *Rhizomucor miehei* are 54% identical. With such an identity percentage, one expects model building by homology to be straightforward, with only a few problems to show up in modeling the surface-exposed loops. Indeed, the sequence alignment shows only two rough spots: around position 74 and around position 257. Inspection of the three-dimensional structure around position 74 makes it clear that the location of the insertion can only be as indicated in the alignment in Figure 8. However, the local structure around residue 74 cannot be built unambiguously.

The location of the indel around position 254 would be hard to find based on sequence information alone. Visual inspection of the structure around this position made clear, however, that the threonine at position 254 is sitting in a beta-bulge. This residue can be deleted with only minimal disturbance of the local environment in the structure. Additional facts in favor of this solution for the local sequence-alignment problem are that the glycine at position 256 ends up favorably in a β-turn and that all residues seem to fit well in their three-dimensional environment.

All lipases of this family are characterized by a helical lid that covers the active site (Fig. 9). It has been hypothesized [145] that this lid moves away in a hydrophobic environment, thereby making the active site accessible to the lipid substrate. In this hypothesis an important role has been reserved for the bulky tryptophane residue at position 117 in the center of the helical lid. In the *Rhizopus javanicus* lipase, however, this bulky hydrophobic residue is replaced by a alanine, and no other bulky residue seems located so that it could take the role of residue 117. Therefore, it was concluded that the lipid-interaction model that has been suggested for this class of lipase is not generally applicable in all its details.

From the model of *Rhizopus javanicus*, suggestions about potential glycosylation sites and likely spots for acidic hydrolysis have been derived [144].

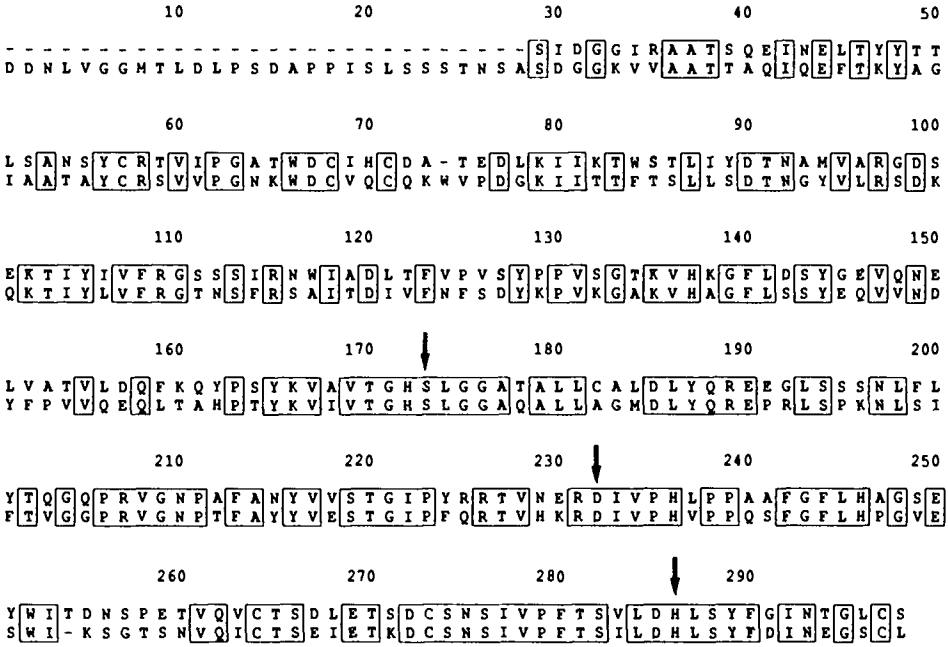

Figure 8 Sequence alignment between the lipases from *Rhizomucor miehei* (top) and the lipase from *Rhizopus javanicus* (bottom). Identical residues are boxed. Arrows indicate the catalytic triad.

IV. ENZYME TARGETS FOR MODELING

There are no limits to what can be modeled, as long as structural information from a homologous molecule is available. Table 3 shows a list of enzymes that are (potential) targets for rational, i.e., structure-based, drug design. Although this list is far from complete, it gives an idea about the field in which molecular modeling can provide a contribution.

APPENDIX

The most important sources of information for molecular modeling are the PDB [115], the SwissProt protein sequence database [138], the Genbank, and the EMBL nucleotide database.

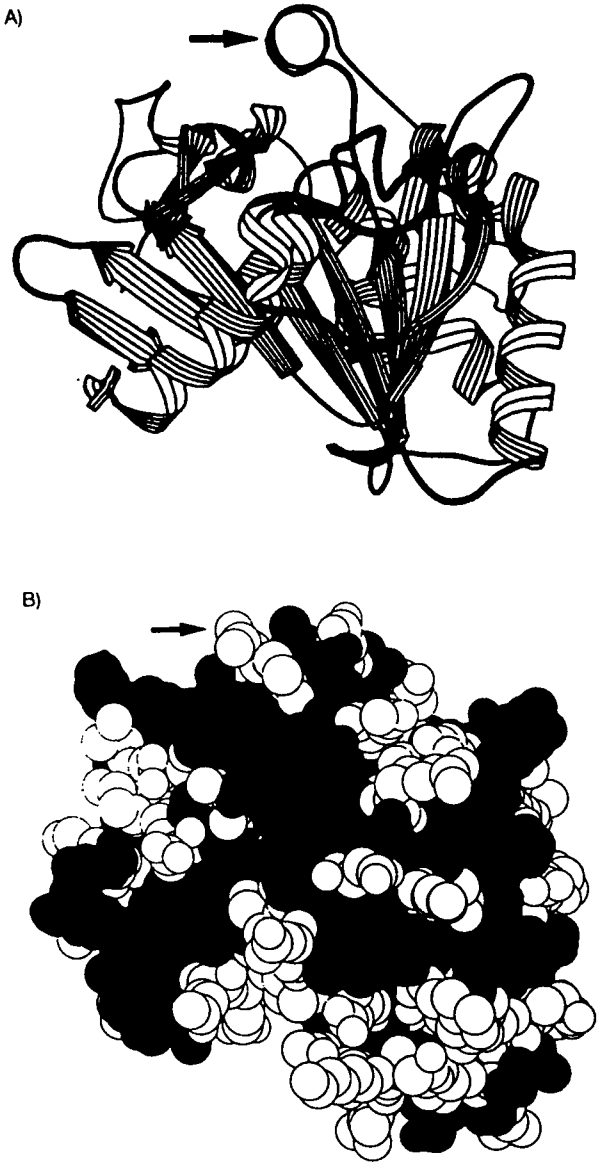

Figure 9 (A) Schematic representation of the model of *Rhizopus lipase.* The arrow indicates the helical lid covering the active site. (B) Representation of the atomic structure of the *Rhizopus lipase* drawn with the Van der Waals radii. The view is the same as that of A. The nonpolar residues are shaded.

Table 3 Some Pharmaceutically Important Enzymes as Targets for Modeling

Enzyme	Therapeutic application
Adenosine desaminase	Severe combined immune deficiency syndrome
Aldose reductase	Diabetes
Angiotensin-converting enzyme	Hypertension
Aromatase	Estrogen-mediated breast cancer
l-Asparaginase	Leukemia
Calcineurin	Immunosuppression
Carbonic anhydrase	Glaucoma, epilepsy
Cholinesterase	Dementia
Coagulation factors	Bleeding disorders
Dihydrofolate reductase	Cancer, bacterial infection
DNA, RNA polymerases	Cancer, viral diseases
Elastase, chymase, cathepsin G	Emphysema, inflammation, connective tissue destruction
Enkephalinase	Analgesia
Enzymes of purine and pyrimidine pathways	Cancer, immunosuppression, metabolic defects
Glucocerebrosidase	Gaucher's disease
HIV aspartate protease	AIDS
H^+, K^+-ATPase	Stomach ulcers
Hydroxymethylglutaryl CoA reductase	Hypercholesterolemia
Interleukin-1 converting enzyme	Inflammation
β-Lactamases	Infection
5-Lipoxygenase	Asthma, arthritis
Lipase (pancreatic)	Obesity
Matrix metalloproteinases (collagenase, gelatinase, stromelysin)	Degenerative tissue diseases, metastasis
Mono amino oxidase A	Depression
Mono amino oxidase B	Dementia
Na^+, K^+-ATPase	Cardiac disorders
Neuraminidase	Influenza
Phosphodiesterase	Inflammation
Phospholipase A_2	Inflammation
Plasmin	Fibrinolysis
Renin	Hypertension
Reverse transcriptase	AIDS
Superoxide dismutase	Oxidative stress, ischemia
Thrombin	Thrombosis
Thromboxane A_2 synthetase	Asthma
Tissue plasminogen activator	Vascular occlusion
Topoisomerase	Cancer
Triose phosphate isomerase	Trypanosomiasis
Trypanothione reductase	Trypanosomiasis
Urokinase	Vascular occlusion

The EMBL in Heidelberg provides a multitude of services that can be helpful for the molecular modeler. DSSP, HSSP, FSSP, PDBFINDER, PDBREPORT, and PDB_SELECT are derived protein databases. They are based on data from the Protein Data Bank of 3D structures and the Swissprot database of 1D protein sequences. The data and related software can be obtained via anonymous FTP or, more easily these days, via the WWW.

DSSP	secondary structures extracted from 3D coordinates
HSSP	sequences homologous to known 3D structures
FSSP	structurally aligned families of proteins
PDBFINDER	short index of PDB files for easy searching
PDBREPORT	lists of all anomalies (and errors!) found in PDB files
PDB_SELECT	representative selection of PDB datasets

These databases can be accessed via the WWW. A good starting address is: http://swift.embl-heidelberg.de

Other services accessible from or pointed at in this WWW page are:

PHD secondary structure prediction
Homology modeling servers
A G protein–coupled receptor database
A mixed pallet of software (including the WHAT IF modeling program)

Commercial users must apply for a license for some of these services.

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The Cysteine Proteinases from the Latex of *Carica papaya* L.

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I. INTRODUCTION

To date, the latex of *Carica papaya* L. is known to contain at least four different proteolytic enzymes, namely, papain (E.C. 3.4.22.2), chymopapain (E.C. 3.4.22.6), caricain or papaya proteinase III or Ω (E.C. 3.4.22.30), and glycyl endopeptidase or papaya proteinase IV (E.C. 3.4.22.25). The importance of the latex of the unripe fruit of the tropical tree *Carica papaya* L. was first noted by G. C. Roy, who in 1873 published in the *Calcutta Medical Journal* (see Ref. 1) an article entitled "The solvent action of papaya juice on the nitrogenous articles of food." The name papain was used for the first time by Wurtz and Bouchet [2] to describe partially purified cysteine proteinases from the papaya latex. They wrote, "*nous désignerons ce ferment sous le nom de papaïne.*" In 1880, Wurtz postulated that papain acts in fibrin digestion by becoming bound to the fibrin [3]. This is remarkable in that Emil Fisher first described the specific association of enzyme with substrate in 1898. Since that time, many names have been used for commercial latex products, e.g., papayotin, papaoid, etc.

In 1937, Balls et al. [4] purified one of the proteolytic fractions and called their crystalline enzyme papain. This was an unfortunate choice, since the name papain is also used for the crude papaya latex containing multiple enzymes. In 1939, Lineweaver and Balls published a refinement of the isolation method, and they also reported the presence in the papaya latex of another proteinase with a higher ratio of milk-clotting to hemoglobin-digesting activity than papain [5]. In 1941 Jansen and Balls presented a description of a second proteolytic enzyme [6]

from the papaya latex. The name chymopapain was chosen following the reasoning of Kunitz and Northrop [7] when they named chymotrypsin, although for chymopapain the higher ratio in fact derived from lower proteolytic activity rather than from higher milk-clotting activity. However, in sharp contrast with papain, chymopapain proved to be a complex mixture of enzymes, and it was a long time before the problems relating to the different molecular species of chymopapain were resolved. Kimmel and Smith reported that chymopapain prepared according to the method of Jansen and Balls was electrophoretically heterogeneous [6]. Ebata and Yasunobu extended the isolation procedure of Jansen and Balls with ion-exchange chromatography to produce an electrophoretically homogeneous enzyme with Glu as its N-terminal residue [8]. In 1967, Kunimitsu and Yasunobu showed that by modification of the former method, two chymopapains could be purified, namely, the enzyme purified in 1962, which they called chymopapain A (according to Cayle et al. [1], who reported in 1962 that he had purified two fractions of chymopapain, namely, chymopapain A and B) and a second enzyme with a higher specific activity toward casein and with Tyr as the N-terminus, which they called chymopapain B.

The interest in papain increased enormously after the publication by Kimmel and Smith [9] of a modification of the purification procedure of Balls and Lineweaver [5]. This modification permitted the isolation of pure papain from papaya dried latex and was used for many years as the standard method for the production of papain. The crystalline papain of Kimmel and Smith consists of three components: active papain, reversibly oxidized papain, and irreversibly oxidized papain. Reversibly oxidized papain can be converted into active papain by reduction of the active-site thiol by low molecular weight thiols [10], sodium borohydride [11], or CN^- [12]. In active papain, the Cys-25, which is essential for catalytic activity, is present in a reduced form, while in reversibly oxidized papain the Cys-25 forms a mixed disulfide with cysteine. Drenth et al. reported that in irreversibly oxidized papain the Cys-25 has been oxidized to the sulfinic acid [13]. When this purification procedure was applied to spray-dried latex, the papain was often contaminated with chymopapain [14].

The method of Kimmel and Smith was modified by Baines and Brocklehurst [14] in order to obtain papain free of any contaminating chymopapain from spray-dried papaya latices. Eighty percent of the papain obtained with the method of Baines and Brocklehurst can be fully activated. For most purposes it is not necessary to carry out the crystallization, since little or no improvement in the purity of papain is achieved by this step.

The amino acid sequence of papain was determined in 1970 by Mitchel et al. [15] (Fig. 1). It exists as an unglycosylated polypeptide of Mr 23406 (212 amino acid residues). Papain was the first cysteine proteinase for which the three-dimensional structure was deduced from x-ray diffraction data. The

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                1         2         3         4         5
      12345678901234567890123456789012345678901234567890123456789
Pap   IPEYVDWRQKGAVTPVKNQGSCGSCWAFSAVVTIEGIIKIRTGNLNEYSEQELLDCDRR
Chp   Y QSI   A           A        TIA V   N  V    L L     V   KH
Car   L  N    K       RH             A V   N     K V L     V  E
Glep  L  S    A        H  Y E      T A V   N  K    V L     V   LQ

      6         7         8         9         10        11        12
      012345678901234567890123456789012345678901234567890123456789
Pap   SYGCNGGYPWSALQLVAQYGIHYRNTYPYEGVQRYCRSREKGPYAAKTDGVRQVQPYNEGA
Chp       K   QTTS  Y  NN V TSKV   QAK YK  ATD PGPKV IT YKR PSNC T
Car    H  K    PY  E   KN   L SK   KAK GT  AKQV GPIV  S  GR   N   N
Glep       R  QSTS  Y   N   LRAK   IAK QT  ANQV GPKV  N  GR  SN   S

               13        14        15        16        17
      12345678901234567890123456789012345678901234567890abcd01234567
Pap   LLYSIANQPVSVVLEAAGKDFQLYRGGIFVGPCGNKVDHAVAAVGYGPN----YILIKNSW
Chp   F GAL    L  LV  G  P    KS V D    T L    T     TSDGKN  I
Car     NA  K      V SK RP    K    E    T      T     KSGGKG
Glep    NA  H      V S  R   N K    E S  T      T     KSGGKG

       18        19        20        21
      890123456789012345678901234567890123
Pap   GTGWGENGYIRIKRGTGNSYGVCGLYTSSFYPVKN--
Chp    PN   K  M L  QS   Q T  V K  Y  F GFA
Car    A    K       AP   P      K  Y  T  --
Glep   P         R AS   P    V R  Y  I  --
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Figure 1 Amino acid sequences for papain (Pap) [15], chymopapain (Chp) [33], caricain (Car) [34], and glycyl endopeptidase (Glep) [45]. A, Ala; B, Asx; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; Z, Glx.

structure of papain has been determined at 1.65 Å resolution [16]. Papain consists of a single polypeptide chain folded to form two domains with a deep cleft between them, containing on one wall the catalytic site cysteine residue (Cys-25) and on the other the catalytic site histidine residue (His-159) partly embedded in a hydrophobic region, part of which consists of a tryptophan residue which is hydrogen-bonded to an asparagine residue. Moreover, papain contains, in addition to the active site cysteine residue, three disulfide bridges. In 1985, Kamphuis et al. presented a detailed comparison of the structures of papain and actinidain [17]. One important observation is that the hydrogen-bonding interactions in the two enzymes are highly conserved and that the spatial positions of the hydrogen bonds in the protein interior are conserved even when individual analogous residues differ. Hence, the secondary structure in these enzymes is well conserved [18].

Caricain was purified for the first time in 1967 by Schack, who called this enzyme papaya peptidase A [19]. Robinson [20] showed it to be one of the components in commercial chymopapain, and the same enzyme was probably called chymopapain C by Clagget et al. [21]. Schack also mentioned a minor but highly active protein component with an intermediate basicity between chymopapain and papaya peptidase A, which was called papaya peptidase B by Lynn [22].

The first attempts to resolve the complexity of the chymopapain fraction, namely, the exact number and nature of the papaya proteinases, were done using ion-exchange chromatography. According to Brocklehurst et al. [23,24], the latex of *Carica papaya* L. contains at least six different proteinases, which can be separated by ion-exchange chromatography on a SP-Sephadex C-50 column. They can be identified by increasing retention time: papain, chymopapain A, different forms of chymopapain B (B1, B2, and B3), and papaya peptidase A. In 1983, Khan and Polgár [25] reported the purification of "chymopapain S" [26]. Buttle and Barrett obtained highly active chymopapain in 1984 [27]. The first step in their purification method was a selective ammonium sulfate precipitation, after which the chymopapain-rich fraction was applied on a Mono S column (FPLC, fast protein liquid chromatography) at pH 5.0 and the enzymes were eluted with an acetate gradient. The chymopapain-containing fractions were combined and applied on the same column at pH 9.5. Further purification was achieved by eluting the enzymes with a NaCl gradient. Papain, two forms of chymopapain, and caricain can be separated from papaya latex by ion-exchange chromatography (Fig. 2). Caricain was called papaya proteinase III, since this was the third papaya endopeptidase eluting from the ion-exchange column. When enzymes from the latex obtained by tapping fruit purchased from the supermarket were isolated, it was found that all chymopapains were immunologically identical [27]. This led Buttle and Barrett to suggest that it would be best

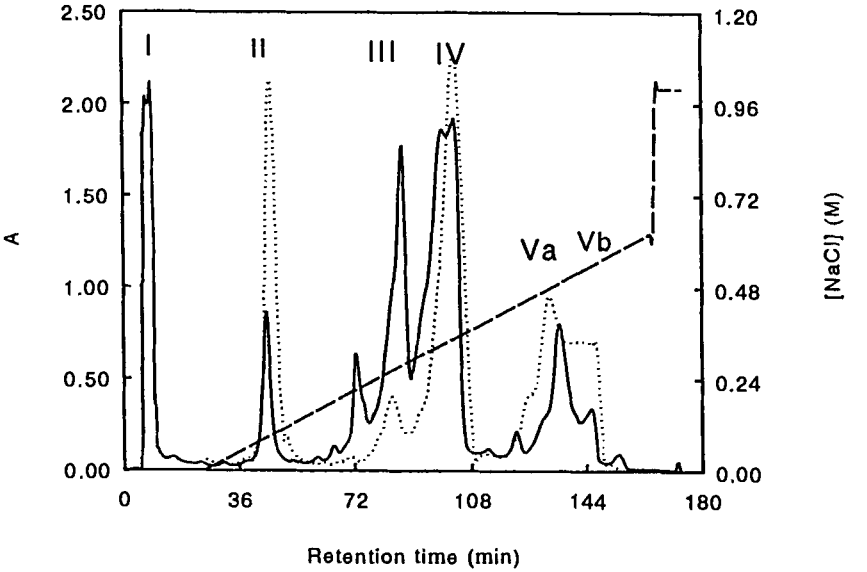

Figure 2 Ion exchange chromatography of the chemically blocked papaya proteinases on a Hi Load S-Sepharose HP 26/10 column. Column: 100 × 26 mm; fractions of 14.4 mL; flow rate 8 mL/min; buffer: 150 mM sodium acetate buffer pH 5.0; elution with a linear gradient of sodium chloride from 0 to 625 mM in 140 min: —— : A280; ..... : A410 (amidase activity against L-BAPNA); – – : sodium chloride gradient; fraction I: unbound material; fraction II: papain; fraction III: mixture of chymopapain A (degradation product) and lysozyme; fraction IV: mixture of chymopapain and papaya proteinase IV; fraction Va and Vb: papaya proteinase III. (From Ref. 42.)

to consider chymopapain as an enzyme produced by a single gene and all other forms of chymopapain as artificial products resulting from the industrial handling of latex.

After Schack named the most basic proteinase of the latex papaya peptidase A, many other names have been used for this enzyme, namely, papaya peptidase II, papaya proteinase III, papaya proteinase A, and papaya proteinase Ω [28]. Most recently, it has been suggested that it should be called caricain [29].

In sharp contrast with some of the results published earlier and mentioned above, the multiple forms of chymopapain isolated by Calam et al. [30] had a single N-terminus, namely, Tyr. They used high-performance liquid chromatography on a TSK SP 5PW column.

Another interesting approach was described by Goodenough and Owen [31]. Unlike most other investigators, they used an anion-exchange column (Mono Q) to separate the proteinases. To achieve separation, all proteinases had to be bound

onto the column at a pH above their isoelectric point. However, the choice of the right pH was limited by the fact that the proteinases precipitated at pH 11.5. Finally, performing the run at pH 10.8, five proteinases could be purified: papain (pI 8.7–8.9; Mr 23 kDa), chymopapain A (pI 10.3–10.4; Mr 24 kDa) and B (pI 10.5–10.7; Mr 24 kDa), papaya proteinase Ω (pI 11.0; Mr 24 kDa), and papaya proteinase β (papaya peptidase B ?) (pI 11.0; Mr 28 kDa).

In 1988, Looze et al. published a series of articles on the cysteine proteinases from papaya latex. In the first article, they described an alternative way to purify chymopapain and papaya proteinase Ω to homogeneity [32]. In the following articles the primary structures of chymopapain [33] and papaya proteinase Ω [34] were described (Fig. 1). The primary structure of chymopapain determined by Watson et al. [35] is in perfect agreement with the results of Dubois et al. Jacquet et al. described the proteolytic specificities of chymopapain and papaya proteinase Ω [36]. The polypeptide chain of caricain contains 216 amino acid residues (Mr 23283). The molecule shares 148 identical amino acid residues (68.5%) with papain, 141 with chymopapain (65.2%), and 175 with glycyl endopeptidase (81.0%). The $A_{1\%,280nm}$ is 18.3 [20]. The three-dimensional structure of caricain has been determined by x-ray diffraction analysis [37,38].

Lynn [22] and Polgár [39] detected in 1979 and 1981, respectively, that the papaya latex contained an endopeptidase with a markedly different specificity from those of the three previously purified enzymes. However, it took until 1989 before this endopeptidase, which has no activity against DL-Bz-Arg-NHPhNO_2, could be separated with high yield from the other papaya proteinases by affinity purification [40]. This enzyme appears to be a major contaminant of chymopapain in papaya latex, as well as in commercial chymopapain preparations (30% of the protein in spray-dried latex). It is very similar to chymopapain in both molecular size and charge, but it differs in the structure of its active center [41]. Glycylendopeptidase can also be separated from chymopapain using hydrophobic interaction chromatography [42] (Fig. 3). The N-terminal 17 residues of glycyl endopeptidase are identical to those published by Lynn and Yaguchi for the enzyme they called papaya peptidase B [43]. These data, as well as the lack of activity against DL-Bz-Arg-NHPhNO_2 and the hydrolysis of Z-Gly-OPhNO_2, are strong evidence that papaya peptidase B and glycyl endopeptidase are the same enzyme. In 1986, McKee et al. [44] had already isolated two cDNA clones from a *Carica papaya* leaf tissue cDNA library. The partial cDNA clone coded for glycyl endopeptidase, while the complete cDNA clone codes for an unknown cysteine proteinase. It is, however, unclear whether this enzyme is present in papaya latex.

The amino acid sequence of glycyl endopeptidase was published by Ritonja et al. [45] (Fig. 1). The enzyme exists as a single unglycosylated polypeptide of 216 amino acid residues (Mr 23.313). Glycyl endopeptidase is clearly a member of the papain family of cysteine proteinases [46]. The three-dimensional structure

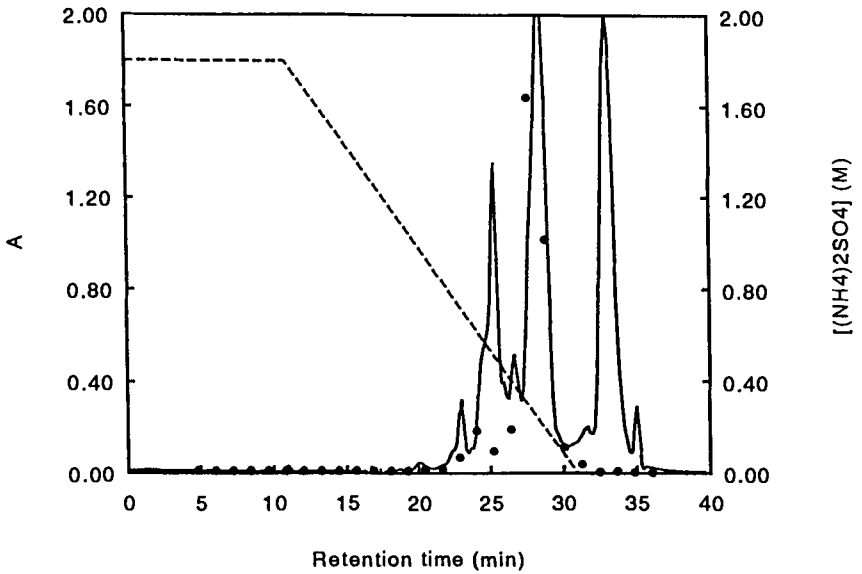

Figure 3 Hydrophobic interaction chromatography of chymopapain (fraction IV from Fig. 1) on a Shodex HIC PH-814 column. Column: 75 × 8 mm; fraction 1.2 mL; flow rate: 1 mL/min; buffer 20 mM phosphate buffer pH 7.0, containing 1.8 M ammonium sulfate; elution: linear gradient from 1.8 to 0 M ammonium sulfate in 20 min; ——: A280; – – –: ammonium sulfate gradient; ●: A410 (amidase activity against L-BAPNA). (From Ref. 42.)

was recently unraveled by O'Hara et al. [47]. This structure demonstrates that the substitution of Gly at position 23 and 65 of papain by Glu and Arg, respectively, is the reason for its abnormal enzymatic behavior. These residues are located in one wall of the active-site cleft, close to the catalytic Cys-25, and their side chains form a barrier across the upper part of the cleft, effectively excluding residues with long side chains from the S_1-binding site.

II. CATALYTIC MECHANISM AND SPECIFICITIES

A. Catalytic Mechanism

A good overview of the enzyme mechanisms involved in proteolysis is given by Antonov [48]. The catalytic action and mechanism are reviewed by Baker and Drenth [49] and by Brocklehurst et al. [50].

Cysteine proteinases contain a catalytically active cysteine sulfhydryl group (Cys-25) and a histidine group (His-159) within the active center of the enzyme [51]. Alkylation of the active-center sulfhydryl group renders the product inactive. The minimal reaction mechanism is represented in Figure 4 [52].

Formation of an enzyme-inhibitor complex is followed by attack by the nucleophylic group of the active site thiol group on the carbon atom of the peptide bond to be cleaved. This results in the formation of a tetrahedral intermediate that is stabilized by interaction of the oxyanion ($C\text{-}O^-$) with the appropriate groups of the enzyme [53,54]. Bond cleavage is completed by donation of a proton (by His-159) to the leaving group. The latter can then diffuse away, leaving an acylated enzyme—in this case a thioester [55]. Upon standing,

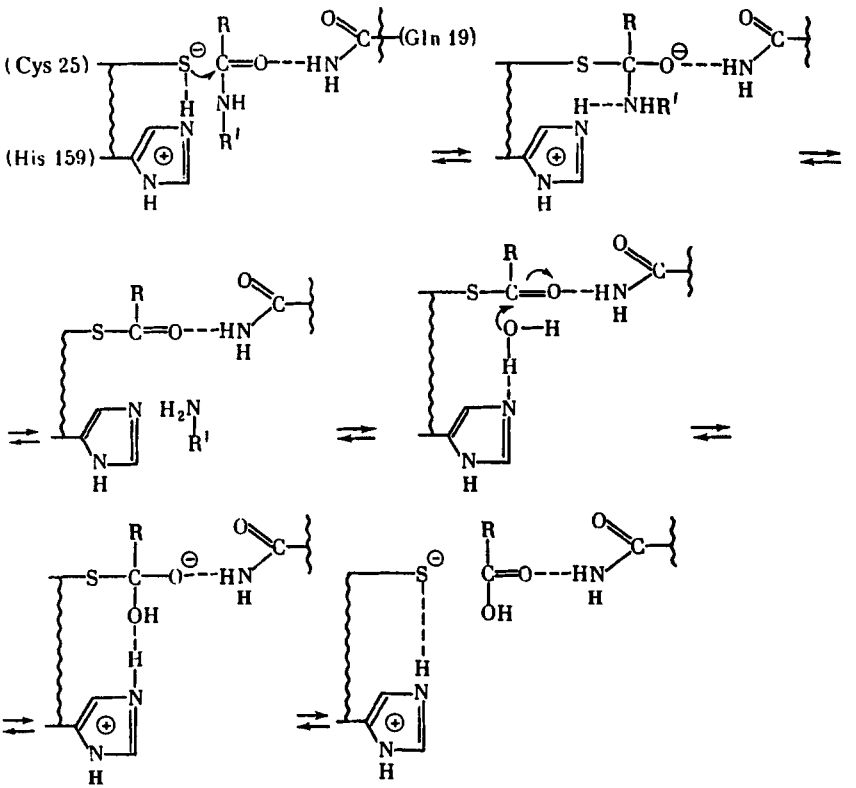

Figure 4 Mechanism of hydrolysis of peptide substrates by cysteine proteinases. (From Ref. 48.)

the acyl group of the thioester is transferred to a water molecule in a slow process to liberate the carboxylic acid and regenerate the active enzyme. Attack of water on the thioester in the deacylation step is catalyzed by the imidazole group of His-159.

Crystallographic studies of native cysteine proteinases and enzyme-inhibitor complexes have been used to interpret much of the kinetic data for cysteine proteinase–catalyzed hydrolysis of amide bonds. Analysis of the crystal structures of papain [16], caricain [38], actinidain [56], etc. shows that these structures are closely related. The active site of all these cysteine proteinases contains the Cys-25 sulfhydryl group in close proximity to the His-159 imidazole ring nitrogens, where the latter can abstract the sulfhydryl proton to facilitate attack on the substrate amide carbonyl group [17].

For a long time it was believed that the Asp-158, which is the only charged group apart from Cys-25 and His-159 in the proximity of the active center, was implicated in the catalytic mechanism of papain [57–59], but x-ray data showed that this group is turned away from the active center to hydrogen-bond with the main chain NH of residue 136 and is consequently too far from the active center to have a direct effect on the mechanism (7.5 Å from Cys-25; 5.5 Å from His-159). Consistent with this is the observation by Barrett et al. [60] that this group is not conserved in the other known cysteine proteinases. Recent work on mutants of papain produced by site-directed mutagenesis, has confirmed this theory, but shows nevertheless that there is an indirect effect of the Asp-158 group on the activity by stabilization of the ion pair by hydrogen bonding [61,62].

Crystallographic studies of papain derivatives whose active centers had been alkylated by reaction with chloromethyl ketones (CK) of di, tri, and tetra peptides provided clear information about the binding modes [63]. The chloromethyl ketones used were Z-Phe-Ala-CK, Z-Gly-Phe-Gly-CK, and Ac-Ala-Ala-Phe-Ala-CK. Some of the postulated interactions in papain-substrate complexes deduced from these studies are shown schematically in Figure 5. These results suggest that a tetrahedral oxyanion intermediate formed in the active center of the enzyme is stabilized by hydrogen bonding to Gln-19 and Cys-25 NH groups. The controversy over the putative role of an oxyanion hole in cysteine proteinases [64,65] has been resolved recently with the kinetic characterization of papain variants by site-directed mutagenesis, which were designed to evaluate the contribution of the Gln-19 side chain to transition state stabilization [66]. The imidazole group is understood to polarize and deprotonate the Cys-25 sulfhydryl group. Due to the ionic form, the imidazolium ion can protonate the nitrogen group of the leaving group while the Cys-25 thiolate attacks the carbonyl atom.

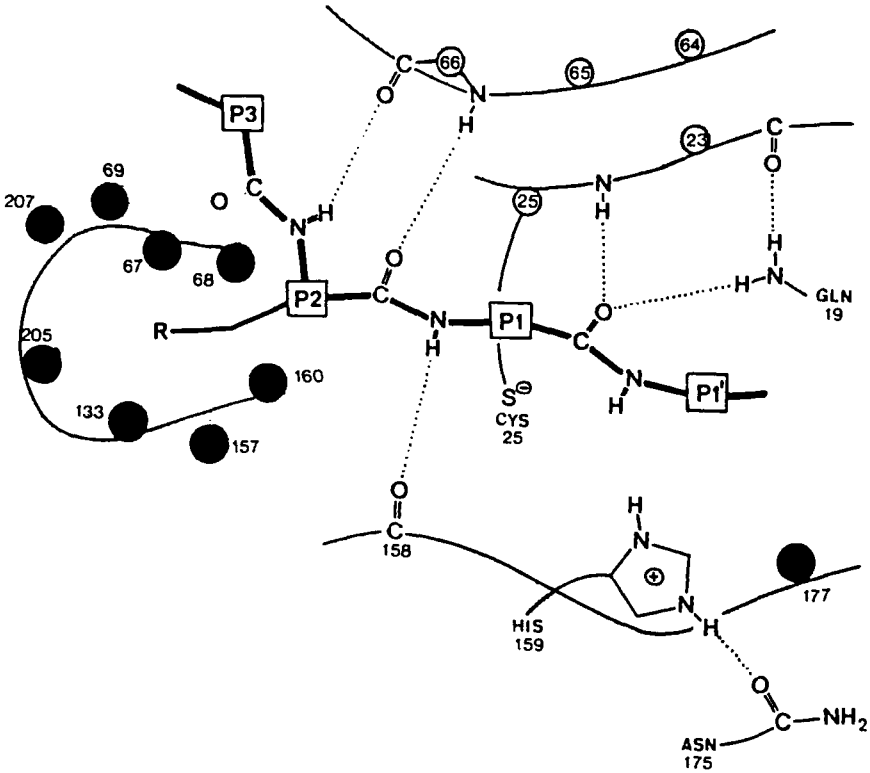

Figure 5 Postulated interactions in a papain-substrate adsorptive complex deduced from crystallographic studies on enzyme-inhibitor combinations. In the enzyme-inhibitor combination, the terminal methylene group of an inhibitor such as Z-Phe-Ala-CK is attached to Sγ of Cys-25, and the carbonyl oxygen adjacent to it (i.e., of P1 C=O which corresponds to the C=O of the scissile peptide bond of a substrate) is directed toward two NH groups. These are the main chain NH of residue 25, which is at the N-terminus of α-helix 24–42, and an NH group of the amide side chain of Gln-19. The peptide between P1 and P2 lies near the extended piece of chain comprising residues 65–67 of the wall of the active center cleft such that the P2 NH and C=O groups can hydrogen-bond with the C=O and NH groups, respectively, of Gly-66 and the P1 NH is directed toward the C=O of residue 158 on the opposite wall of the cleft. The side chain of residue P2 (Phe) is accommodated so that the Cβ lies between the side chains of Pro-68 and Ala-160, and beyond it the phenyl ring is adjacent to the side chains of Val-133 and Val-157, which with Tyr-67, Trp-69, and Phe-207 form the hydrophobic binding pocket. (From Ref. 18.)

B. Specificities

It is well known that the cysteine proteinases of the papaya latex differ in their ability to hydrolyze proteins and synthetic peptides. Schechter and Berger [67] first demonstrated that the binding regions in the active center of proteinases can be divided into different subsites, of which the S_2 subsite seems to be the most important in papain.

Specificity is often described in terms of the subsite model of Schechter and Berger [67,68] (Fig. 6). In terms of this model, the substrate may be represented as P_4-P_3-P_2-P_1-↑-P'_1-P'_2, where P represents an amino acid residue that binds to a particular region in the active center (subsite S_1,S'_1, etc.) and the arrow indicates the scissible bond, of which P_1 provides the carbonyl moiety and P'_1 the amino moiety. The reactivity and inhibition studies of Berger and Schechter [68] showed that papain has a strong affinity for Phe, Tyr, Val, or Leu at P_2 of the substrate. A similar preference is shown by all papaya proteinases, although significant differences exist.

The residues in the S_2 subsite that make the most intimate contact are those of Val-133, Val-157, Ala-160, Pro-68, and Tyr-67. In the four enzymes of the papaya latex, all these groups remain hydrophobic, with the exception of Pro-68, which is replaced by Glu in chymopapain and in glycyl endopeptidase. The nonpolar character, and generally specificity, of the S_2 subsite would therefore be conserved.

The S_1 subsite has no binding pocket as such and has a much less clearly defined specificity, expect for glycyl endopeptidase. Low molecular weight substrates indicate some preference for Lys or Arg (over Ala) at P_1 of papain [3]. Schechter and Berger [68] showed that Leu and Phe bind equally well at S_1, indicating that the interaction is probably mainly hydrophobic. It is striking, however, that Val is not accepted in S_1 [49].

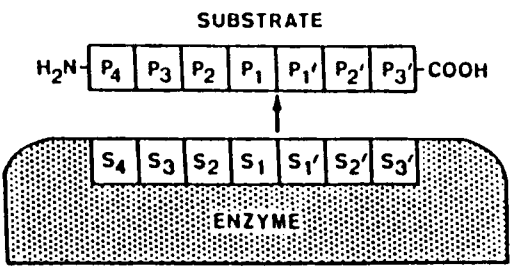

Figure 6 The active site of papain according to Schechter and Berger [67].

In glycyl endopeptidase, however, the Gly-23 and Gly-65 of papain are replaced by Glu and Arg, respectively [45]. These residues are located on the side walls of the active site cleft and can thus sterically hinder the interactions at the S_1 subsite, which is only accessible for Gly in glycyl endopeptidase. The absolute requirement for L-amino acids at P_1 arises from the fact that when a peptide binds to the enzyme the hydrogen on the α-carbon points toward the enzyme (in the vicinity of Gly-23) [63]. This is probably the reason why D-amino acids cannot bind properly (their side chain would clash with Gly-23) and why Gly is always conserved in position 23.

III. APPLICATIONS OF THE PAPAYA ENZYMES

A. Industrial Applications

Crude papaya latex, usually called "papain," has found a number of industrial applications. It has been used in brewing, meat tenderization, flavor production, fish processing, cheese production, and the baking and pharmaceutical industries [50,69]. The use of papain, however, differs greatly from country to country; e.g., in France papain is used almost exclusively in the fish industry, while in the United States 50% of papain is used for the tenderization of meat. Although the brewing industry is the major market for papain (±75%), its use in beer for local consumption is forbidden in some countries, while in other countries there are legal restrictions on its use for meat tenderization

Papain is used in the brewing of beer in the mashing process to allow high levels of barley versus malt to be used as well as in the chill-proofing of clear beer to prevent the formation of a chill-haze on cold storage. It was used for the first time in 1911 for the chill-proofing of beer; this represents the first industrial application of enzymes. Due to the limitations imposed by legislation in some countries, immobilized papain has met increasing success in the chill-proofing of beer.

The use of papain in meat tenderization [70] is almost completely restricted to the United States. A number of techniques have been described (preslaughter as well as postslaughter administration) but are not always very successful. In all cases, papain action does not occur before the cooking of the meat and therefore the result is influenced by many factors other than the actual method of application. It has also been argued that the use of papain in meat tenderizers, toothpaste, beer, and fruit juices is the main reason for the higher incidence of systemic allergic reactions after injection of chymopapain for treatment of sciatica in North America than in Europe.

B. Medical Applications

1. Chemonucleolysis

a. Introduction

The use of chymopapain for the treatment of sciatic has been extensively reviewed by Nachemson and Rydevik [71] and by Buttle [72]. Chemonucleolysis is the name used for the treatment of sciatica pain by injection of chymopapain into a protruded or herniated lumbar intervertebral disk.

Thomas described for the first time the effect of crude papain on cartilage [73]. The intravenous injection of papain into young rabbits resulted in loss of meta-chromasia in all the cartilagineous tissues in the body, demonstrated by collapse of the rabbits ears. In 1964, Smith introduced chymopapain as a possible treatment for sciatica and called this procedure chemonucleolysis [74]. The concept of injecting a proteolytic enzyme into a disc for treatment of disc disease was, however, first suggested by Hirsch [75]. His hypothesis was that the intradiscal injection of a proteolytic enzyme would speed up the normal process of disc degeneration to an end stage, where the proteoglycan concentration of the disc is reduced and the disc has become stable and asymptomatic. The drug has since then been under study, but it was not until November 1982 that chymopapain (Chymodiactin®-Smith Laboratories and Discase®-Baxter-Travenol) was approved by the U.S. Food and Drug Administration and was distributed worldwide as a chemonucleolytic drug.

Chymopapain (a mixture of proteinases without papain) is thought to act by degrading the proteoglycan component of the nucleus pulposus. Its action is often explained as an attack of the highly positively charged proteinase on the highly negatively charged proteoglycans. However, Buttle et al. were able to demonstrate that modified (acidic) forms of chymopapain effectively released glycosaminoglycans from bovine nasal cartilage effectively [76]. Evidence for the action of the papaya proteinases on the proteoglycan fraction of the intervertebral disk is given by the fact that after intradiscal injection of even low doses of chymopapain, proteoglycans are broken up into smaller fragments of glycosaminoglycans and water, which diffuse out and can be detected in urine [77]. Some reports indicate that the degree of tissue damage is dose-related; doses from 100 µg up to 10 mg produce proportionally more damage [78]. Dando et al., however, reported that the total excretion of glycosaminoglycans during a 5-day period after injection was independent of the initial dose [79]. Collagen, the second constituent of the nucleus pulposus, remains unaffected by the action of chymopapain [80]. Although animal studies have shown that the effect of chymopapain injection is reversible [81], it has been shown by many authors that the disc height decreased after chymopapain injection in humans [82].

b. Toxicity and allergenicity

Before discussing the efficacity of chemonucleolysis, it is important to consider some of the side effects of this type of treatment. An enormous amount of literature dealing with such problems has been published, and ever since its introduction there has been intensive debate on the subject of treating sciatica by chemonucleolysis. The most important complication is anaphylaxis. It is, however, interesting that the incidence of IgE-mediated allergic reactions in North America is higher than in western Europe (about 1% in the United States and 0.2% in Europe) [83,84]. This might be explained by a higher exposure and sensitization to chymopapain because of its frequent use in meat tenderizers, toothpaste, beer, and fruit juices in North America. Sagona et al. [85] demonstrated that IgE and IgG with reactivity against chymopapain can be introduced by intradiscal injection of chymopapain and therefore multiple injections cannot be considered [86]. Preinjection screening to detect and exclude patients at risk has been reported, and skin testing proved to be more accurate than in vitro testing for specific IgE determinants [87].

It has also been argued that chymopapain injection under local, rather than under general, anesthesia is associated with a lower risk for anaphylaxis [88]; another advantage of chymopapain under local anesthesia is the fact that the patient will react if the injection needle comes in close contact with the nerve root [89]. Indeed, injection of chymopapain into the nerve root must be avoided because chymopapain can cause neural injury if it comes in direct contact with the nervous tissue.

Since there is always a potential risk that epidural leakage of chymopapain from the disk space will occur, there is a chance that other tissues, such as epidural fat, dura, and local nerve endings, will be exposed to proteolytic enzyme. A number of neurological complications have been described, including subarachnoid hemorrhage [90], paralysis, and cauda equina compression syndrome [91].

c. Clinical results

Although chemonucleolysis has been used for more than 25 years as an alternative treatment for sciatica, there has always been controversy as to its use, and to date many physicians are still reluctant to use it. Because of the controversial results, it seems desirable to take a closer look at this important feature. In the first clinical series of 75 patients published, 75% good results were reported, although 39% of patients had severe muscle spasms and an increase in back pain for a few days [92]. This phenomenon was also reported by others. In recent clinical trials (properly controlled, double-blind prospective studies), an overall success rate of about 70–85% has been reported [93–95].

One important problem in determining efficacy of treatment seems to be the choice of the type of control. While in most cases the same volume of saline is injected into the intervertebral disk as a control [96], little or no data are available that compare the effect of chymopapain treatment with the natural history of the disease [97]. This problem was discussed in an editorial in the *Lancet* in 1986 [98]. However, a number of studies have been published that compare chemonucleolysis with traditional surgical disc removal [99–102]. These studies indicate that surgical disc removal has an equal or slightly better overall success rate than chemonucleolysis. It has been argued that if chymopapain treatment fails, subsequent surgery may be less successful, especially if the operation is delayed [103], but recent results indicate that this is not the case [104].

Proper patient selection seems to play a crucial role in the outcome of chemonucleolysis [105]. There are a large number of conditions that can disqualify a patient, such as previous laminectomy, rapidly progressive neurological deficit, especially with involvement of bladder and bowel dysfunction, spinal tumor, spinal stenosis, spondylolisthesis, multiple level disc herniation, severe allergy, diabetes, and pregnancy. Absent all of these conditions, there are still a large number of cases where it is difficult to decide on the suitability of chymopapain treatment. Although improvements in the preinjection evaluation have been made [106], further improvements in patient selection seem to be essential to the future success of chemonucleolysis.

f. Toward a safer treatment of sciatica, a look in the future

Although chemonucleolysis with Chymodiactin®-Discase® can now be regarded as a valid alternative to classical surgical disc removal, scientists and physicians are still working to improve this type of treatment. Since there are two basic problems—allergic reactions and neurological complications—several alternatives can be envisaged.

Several reports have been published comparing the effects of chymopapain with other agents thought to be possible alternatives for chemonucleolysis. In 1987 and 1992, Hedtmann reported the results of prospective, comparative studies of intradiscal high-dose and low-dose collagenase versus chymopapain [107,108]. His reports indicate that the overall success rate after treatment with collagenase is lower than after treatment with chymopapain. Moreover, Takenaka et al. demonstrated that collagenase is not only effective against the nucleus pulposus, but also acts on the annulus fibrosus and the end-plates [109]. They therefore considered treatment with collagenase as dangerous for use in humans. Hyaluronidase, however, acted only on the nucleus pulposus and was regarded as a potential synergistic drug for chemonucleolysis.

Another approach was mentioned by Dando et al. [110]. Replacing chymopapain with human proteinases could reduce the risk of allergic reactions. Dando et al. demonstrated that cathepsin B and cathepsin G are capable of reducing the intervertebral disc height in rabbits. Therefore, cathepsin B could be a safer drug for chemonucleolysis, since it is not a foreign protein and moreover not active at neutral pH. A similar approach was followed by Wakita et al. [111]. These authors used calpain I as a proteolytic enzyme. The obvious advantage of this choice is the fact that calpain I can be purified from red blood cells and does not require the use of recombinant DNA techniques.

A more simple and straightforward approach for the improvement of chemonucleolysis could be found in the further purification of the chymopapain preparation. Since Chymodiactin®-Discase® contains ±70% chymopapain and up to 30% other proteins and proteinases, it would be reasonable to inject a 99% pure chymopapain preparation. Dando et al. have shown that contaminating enzymes (20% caricain and 4% glycyl endopeptidase) account for 75% of the total IgE antibodies against cysteine proteinases from papaya latex in persons with allergic reactions after chemonucleolysis [112]. In order to assess whether a mixture of proteinases, as is present in Chymodiactin®, could be replaced by affinity purified chymopapain preparation, we compared the rates of degradation of purified monomeric aggrecan, believed to be the main substrate in vivo [76] by all four papaya proteinases [113]. The rate of degradation of aggrecan by pure chymopapain was not significantly different from the rate of degradation by a mixture of proteinases resembling the proteinase composition of Chymodiactin®. We concluded that purified chymopapain could replace Chymodiactin® for the treatment of sciatica.

Finally, an article by Grubb should be mentioned in which he stated that the use of cysteine proteinase inhibitors, such as cystatin C and kininogens, could possibly reduce the risk of neurological complications such as cerebral hemorrhages [114].

2. Other Medical Applications

In eastern Europe, Lekozim®, a mixture of the papaya proteinases, is used for the treatment of eye burns [115] as well as for some cases of cataract [116]. Recently, a new application for chymopapain has been reported. Chymodiactin® has been used in the process of removal of tumor cells from autologous marrow grafts in a positive selection system [117,118]. This process clears the way for the use of autologous bone marrow transplants in the treatment of several types of cancer, since the authors showed that hemopoietic stem cells, the precursors of all lymphohemopoietic lineages, are present in cell mixtures purified in this way [119].

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Bromelain

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I. INTRODUCTION

Bromelain is the collective name for closely related proteolytic enzymes found in tissues of the plant family Bromeliaceae, of which pineapple, *Ananas comosus,* is the best known. Two distinct types of pineapple bromelain are recognized: stem bromelain (EC 3.4.22.32), the major proteolytic cysteinyl protease in pineapple stem, and fruit bromelain (EC 3.4.22.33), formerly called bromelin, which is the major proteolytic component in pineapple fruit [1]. Stem bromelain has been known under a variety of synonyms, namely, pineapple stem bromelain, EC 3.4.4.24 (formerly), EC 3.4.22.5 (formerly), included in EC 3.4.22.4 (formerly), ananase, bromelase, pinase, extranase, traumanase, pineapple enzyme, and juice bromelain.

Bromelain enzymes have a long history in medicine. The use of pineapple tissue as a folk medicine in Central and South America was reported by explorers in the seventeenth century [2]. In 1558 Thévet wrote: "the fruit of which the natives of America make the greatest medicinal use is called nana (ananas, pineapple). It admirable recreates and exhalirates the Spirites and conforts the Haert, it also fortifies the Stomack, cureth queasiness and causeth Appetite, it gives present ease to such as are troubled with the Stone of stoppage of urine. If the fruit is not procurable the root will have the same effects" [2,3].

The older literature concerning the medicinal use of bromelain has been summarized by Moss [4]. Reviews up to 1976 are cited by Taussig et al. [5], Dupaigne et al. [6], and Cooreman et al. [7].

Considerable research has been carried out during the last two decades investigating the clinical effects of bromelain. The bromelain preparations used in most of these studies were not homogeneous. Crude bromelain is a mixture of different substances, each of which may contribute to the pharmacological action. As we shall see, few studies define the biochemical composition of the bromelain preparations used in clinical and pharmacological investigations. Interpretation of the results is often very difficult, especially the unequivocal allocation of an observed pharmacological effect to the proteolytic enzyme in the mixture. In most studies "bromelain" indicates the proteolytic enzyme or a preparation containing different components from the pineapple plant. Evidence is given that the pharmacological action is connected, but not solely due to, the proteolytic activity of the preparation.

II. COMPOSITION OF CRUDE STEM BROMELAIN

Since heterogeneity of stem bromelain was reported by Heinicke and Gortner [8], a number of studies have indicated that crude stem bromelain contains several proteolytically active components together with some nonproteolytic enzymes, inhibitors, and activators [9–31]. An extensive review of the purification procedures of different proteolytic components of crude stem bromelain up to 1976 is given by Cooreman et al. [7].

Two fractions with similar proteolytic activities and molecular properties except for their electric charge were first separated by anion exchange. The major enzyme fraction, stem bromelain, had an isoelectric point of 9.55, an amino-terminal Val residue, and a molecular weight of 33,200 obtained by sedimentation and diffusion experiments [10,14]. Two isoenzymes of bromelain were separated by isoelectric focusing [12], resulting in a major basic component with an isoelectric point of 9.45 and a minor acidic component with an isoelectric point of 4.7. The basic glycoprotein with N-terminal Val or Ala and a molecular weight of approximately 28,000 is designated stem bromelain (EC 3.4.22.32), while the acidic protein with molecular weight of 18,000 and N-terminal amino acid Ala is designated fruit bromelain (EC 3.4.22.33).

A detailed study concerning the occurrence of different proteolytic enzymes in crude stem and fruit bromelain was carried out by Ota et al. [15]. Fractionation of crude stem bromelain and characterization of the components revealed six proteolytically active components (SBB 1–5 and SBA) [15,16]. All active fractions were analyzed by SDS PAGE and migrated as a single band with molecular weights of 27,000 for SBB 1–3 and about 23,000 for SBB 4 and 5. Slight differences among SBB 1–3 were observed in a few amino acid contents and in

the amount of extraneous end group. SBB 4 and 5 moved slightly faster on SDS PAGE than SBB 1–3, contained more Arg, less Lys, Asp, Glu, and Ala, and were more active against casein and some synthetic substrates [16]. Harrach et al. detected by two-step FPLC cation exchange chromatography in crude stem bromelain at least eight basic proteolytically active components with slight differences in molecular weight. One of the components, representing 2% of the total protein, has a 15-fold higher specific activity toward the substrate L-pyroglutamyl-1-phenylalanyl-1-leucine-*p*-nitroanilide than the main component and was found to be unglycosylated [25]. Stem bromelain was isolated from acetone powder using affinity chromatography on a column of ε-aminocaproyl-D-tryptophan methyl ester coupled to sepharose 4B [17]. The isolated enzyme showed a molecular weight of 23,000 and could be converted to two chains of 15,000 and 8,500 upon reduction.

Pure stem bromelain with high catalytic activity was obtained by an elegant isolation procedure using solubilization in the presence of Amberlite XAD-4, DEAE chromatography and bioaffinity chromatography on agarose-1,6-diaminohexane-*p*-chloromercuribenzoate [18–20].

Rowan et al. [21–23] isolated two previously unknown cysteine proteinases from pineapple stem, called ananain (EC 3.4.22.31) and comosain, by active-site directed affinity chromatography on Sepharose-Gly-Phe-glycinaldehydesemicarbazone or Sepharose-Phe-glycinaldehydesemicarbazone and cation exchange chromatography. The relative molecular mass of ananain (25,000) is very similar to that of stem bromelain (26,000), but ananain shows different specificity for peptide and protein substrates. Ananain has a broad substrate specificity, with benzoyl-Phe-Val-Arg-7(-4-methyl)coumarinylamide being the most susceptible for cleavage by ananain (k_{cat}/K_m 4870 $mM^{-1}s^{-1}$). Ananain is strongly inhibited by chicken cystatin and by compound E-64 and is immunologically distinct from bromelain. Ananain contains five isolatable alternative forms, which were characterized by amidolytic activity, thiol stoichiometry, and accurate mass determinations [24]. The amino acid composition and kinetic specificity toward E-64 of comosain differs from that of stem bromelain [24]. Comosain is also clearly different from stem bromelain in its substrate affinity. The Michaelis constant K_m of comosain for *N*-benzyloxycarbonyl-Arg-Arg-7(-4-methyl)coumarinylamide (1.4 μM) shows that this proteinase is distinct from stem bromelain (K_m 15.4 μM) and ananain (K_m 44.5 μM) [22].

Several nonproteolytic enzymes have been isolated from crude stem bromelain including a phosphatase, a peroxidase, a cellulase, and other glycosidases [8,26,27].

A fibrinolysis enzyme activator was isolated from commercial bromelain [28]. The active substance has no intrinsic fibrinolytic activity, nor any

proteolytic activity, but appears to enhance the activity of one of the fibrinolytic enzymes. The presence of this activator could account for the fibrinolytic effect of commercial bromelain.

A family of cysteine proteinase inhibitors different from the cystatin superfamily was isolated from pineapple stem acetone powder. These inhibitors have a Mr of about 5800 and are composed of a longer (41 amino acids) and a shorter (11 amino acids) peptide chain connected with disulfide bonds [29]. The conserved sequence Gln-Val-Val-Ala-Gly of the cystatins is not present in these inhibitors, indicating a different mechanism of interaction. The bromelain inhibitor VI was found to share similar folding and disulfide bond connectivities with the Bowman-Birk trypsin/chymotrypsin inhibitor from soybean [30,31]. The physiological role of these inhibitors remains unclear.

III. SUBSTRATE SPECIFICITY

Stem bromelain has a broad substrate specificity, close to the specificity of ficin (EC 3.4.22.3), and hydrolyzes a great variety of synthetic and natural substrates. It preferentially cleaves peptide bonds when a hydrophobic group is in the P2 position. (According to Schechter and Berger [32], the amino acid residues in a substrate undergoing cleavage are designated as P1, P2, P3, etc. in the N-terminal direction and P1′, P2′, P3′, etc. in the C-terminal direction from the cleaved bond.) Predominantly Gly-Phe, Phe-Ser, Tyr-Ile, and Tyr-Val bonds are cleaved [33,34]. The specificity of bromelain is different from that of papain and ficin in that it hydrolyzes most effectively derivatives of Lys, Ala, Tyr, Gly, and Asn [35].

Stem bromelain is able to interact with two or more sequential amino acids in a peptide substrate, demonstrated by using *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester [36]. A glutamic acid residue in position P3′ prevents cleavage of the susceptible peptide bond. The sequences containing negatively charged residues in position P3′ and P4′ remain intact [37].

The most used synthetic substrates are *N*-benzoyl-L-Arg-ethyl ester (BAEE), *N*-benzoyl-L-Arg-amide (BAA), *N*-benzyloxycarbonyl-Lys-*p*-nitrophenyl ester, *N*-benzoyl-L-Arg-*p*-nitroanilide (L-Bapa), tosyl-L-Arg-methyl ester (TAME), *N*-benzoyl-L-Arg-methyl ester (BAME), Gly-ethyl ester, His-ethyl ester, benzoyl-Gly-ethyl ester, L-Leu-*p*-nitroanilide (Leupa), L-Ala-*p*-nitroanilide (Alapa), L-Phe-*p*-nitroanilide (Phenpa), *N*-benzyloxycarbonyl-Arg-Arg-*p*-nitroanilide and *N*-benzyloxycarbonyl-Arg-Arg-7-(4-methyl)coumarinylamide. Substituted benzoylglycine-methyl ester (substituted in position 3 or 4 of benzene, e.g., 3-cyano hippuric acid methyl ester, and *N*-succinyl-Ala-Met-S-benzyl-Cys-*p*-nitroanilide are also sensitive substrates for assaying bromelain [14,19,24,33,35,38–41] (Table 1).

Table 1 Kinetic Parameters of Substrate Hydrolysis by Stem Bromelain

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	Ref.
Benzoyl-Arg-amide	4.0			33
	3.1	0.0046	0.0015	15
	1.2	0.0035	0.0029	40,42,45
Benzoyl-Arg-ethyl ester	43			33
	190	0.42	0.0022	14
	200	0.73	0.0036	52
	170	0.5	0.0029	40,42
Benzoyl-Gly-ethyl ester	200	0.21	0.0011	14
	210	0.36	0.0017	40,52
Benzoyl-Ser-methyl ester	0.35–3.23			43
Benzoyl-Arg-methyl ester	32	0.11	0.0034	40
Tosyl-Lys-methyl ester	84	0.035	0.0004	40
N-benzyloxycarbonyl-Phe-Ser-methyl ester	0.7–2.0			35
	0.53	3.4	6.41	43
N-benzyloxycarbonyl-Lys-*p*-nitrophenyl ester	0.057–0.082			35
N-benzyloxycarbonyl-Ala-*p*-nitrophenyl ester	0.024–0.049			35
N-benzyloxycarbonyl-Arg-Arg-methylcoumarinylamide	0.015	27.3	1770	21
N-benzyloxycarbonyl-Arg-Arg-*p*-nitroanilide	1.0	0.13	0.13	24
N-benzyloxycarbonyl-Phe-Arg-methylcoumarinylamide	0.083	0.136	1.64	21
Benzoyl-Phe-Val-Arg-methylcoumarinylamide	0.0097	0.893	92.1	22
Benzoyl-Phe-Val-Arg-*p*-nitroanilide	0.074	3.30	45	24

Bromelain differs from the other cysteinyl proteases papain and ficin in its 140-fold difference of k_{cat} for the BAEE and BAA hydrolysis, suggesting a difference in the mechanism of catalysis for both substrates [37]. For BAEE hydrolysis, deacylation is predominantly the rate-limiting step, while for BAA hydrolysis the acylation is rate limiting [42]. However, Wharton et al. [43] explained the differences in k_{cat} for BAEE and BAA hydrolysis assuming that nonproductive binding plays a role in catalysis.

Casein and hemoglobin are the most important natural substrates. Other natural substrates include insulin A and B chain, glucagon, angiotensin, bradykinin, collagen, elastin, ovalbumin, and somatotropin. In glucagon, bromelain cleaves either the Arg^{18}-Ala^{19} or the Ala^{19}-Gln^{20} bond, but never at both sides in the molecule, to liberate free alanine [10]. The Arg^{17}-Arg^{18} and Lys^{12}-Tyr^{13} bonds of glucagon are not cleaved by bromelain. The internal sequence Phe^{22}-Val^{23} of glucagon is left intact, while in the B chain of insulin the N-terminal Phe^{1}-Val^{2} bond is cleaved [34].

The His-Leu bond of the insulin B chain is cleaved within the sequence Ser^{9}-His^{10}-Leu^{11}-Val^{12}, but not in the sequence Gln^{4}-His^{5}-Leu^{6}-Cys^{7}, because of the negative charge in the P2 position. Likewise, the Val-Glu bond is left intact by bromelain in the sequence Ile^{2}-Val^{3}-Glu^{4}-Gln^{5}-Cys^{6}-Cys^{7} of the A chain of insulin because of the negative charges in the P3′ and P4′ position. The same bond is cleaved in the sequence Leu^{11}-Val^{12}-Glu^{13}-Ala^{14}-Leu^{15} of the insulin B chain. Like papain, cathepsin L, and ficin, the Asn^{3}-Gln^{4} and the Glu^{13}-Ala^{14} bonds of the B chain of insulin are effectively cleaved by bromelain. Although the specificity of bromelain strongly resembles that of ficin, the peptides produced after cleavage of the oxidized B chain of insulin by both enzymes are different. The Leu^{6}-Cys^{7} and Gly^{20}-Glu^{21} bond of insulin are cleaved by ficin, but not by bromelain, while for the Tyr^{26}-Thr^{27} bond, the reverse is true [34]. The vasoactive peptide angiotensin I (Asp-Arg-Val-Tyr-Val-His-Pro-Phe-His-Leu) is cleaved at the Tyr^{4}-Val^{5} bond. The nonapeptide bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) is cleaved at the Gly^{4}-Phe^{5} as well as at the Phe^{5}-Ser^{6} bond [34]. In the sequence of the growth hormone somatotropin, bromelain cleaves the Thr^{135}-Gly^{136}, the Ser^{144}-Lys^{145}, and the Lys^{145}-Phe^{146} bonds. Limited proteolysis of plasma kininogen with bromelain releases a fragment of Mr about 22,000 that accounts for all the inhibitory activity of the parent molecule for papain [44].

IV. MOLECULAR PROPERTIES

A. Physical Properties

Ritonja et al. [45] revealed the complete amino acid sequence of stem bromelain. Their results show that stem bromelain exists as a single polypeptide chain with

211 or 212 residues, depending on the presence or absence of the N-terminal Ala (Fig. 1). Based on the amino acid composition, the molecular weight of the larger form of bromelain was calculated to be 22,828 daltons. Stem bromelain is a glycoprotein with one oligosaccharide moiety per molecule, which is covalently attached to the peptide chain [11,46]. The amino acid sequence that attaches the sugar residues is Asn-Asn^{117}-Glu-Ser [47,48] (numbering referring to bromelain sequence in Figure 1). The total molecular weight of the glycosylated form of bromelain, with an oligosaccharide of 1000 daltons [49], should thus be about 23,800. The oligosaccharide chain contains fucose, mannose, xylose, and *N*-acetylglucosamines [11,25,47]. Several partial structures of the carbohydrate chains were proposed [47,50,51] before Ishihara et al. [49] revealed the complete structure of the carbohydrate moiety of stem bromelain. They released the oligosaccharides from the glycopeptides by digestion with almond glycopeptidase, which cleaves β-aspartylglycosylamine linkages in glycopeptides with oligopeptide moieties. The released oligosaccharides were separated by paper chromatography into two components with an integral number of mannosyl residues. The proposed structures for both oligosaccharides are shown in Figure 2.

The structure of stem bromelain oligosaccharides shows interesting features: fucose is attached to the *N*-acetylglucosamine by the α1-3 bond, while the mode of linkage normally is 1-6, and the oligosaccharides contain a xylose residue linked to a β-mannosyl residue by β1-2 linkage. Occurrence of xylose is very rare in Asn-linked sugar chains, and it was the first case in which the mode of linkage of a xylosyl residue was established [49]. The carbohydrate moiety of stem bromelain seems not to be directly involved in the catalytic activity since

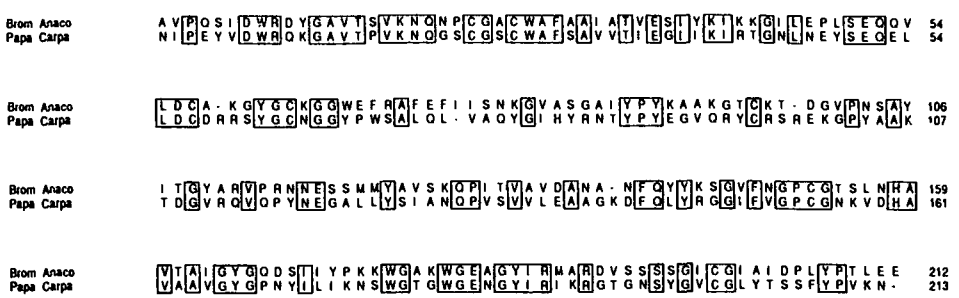

Figure 1 Alignment of the amino acid sequences of stem bromelain and papain. Amino acids identical for both sequences are boxed. Brom Anaco: Bromelain from *Ananas comosus*; Papa Carpa: papain from *Carica papaya*. (Sequences were extracted from the SwissProt protein sequence data base.)

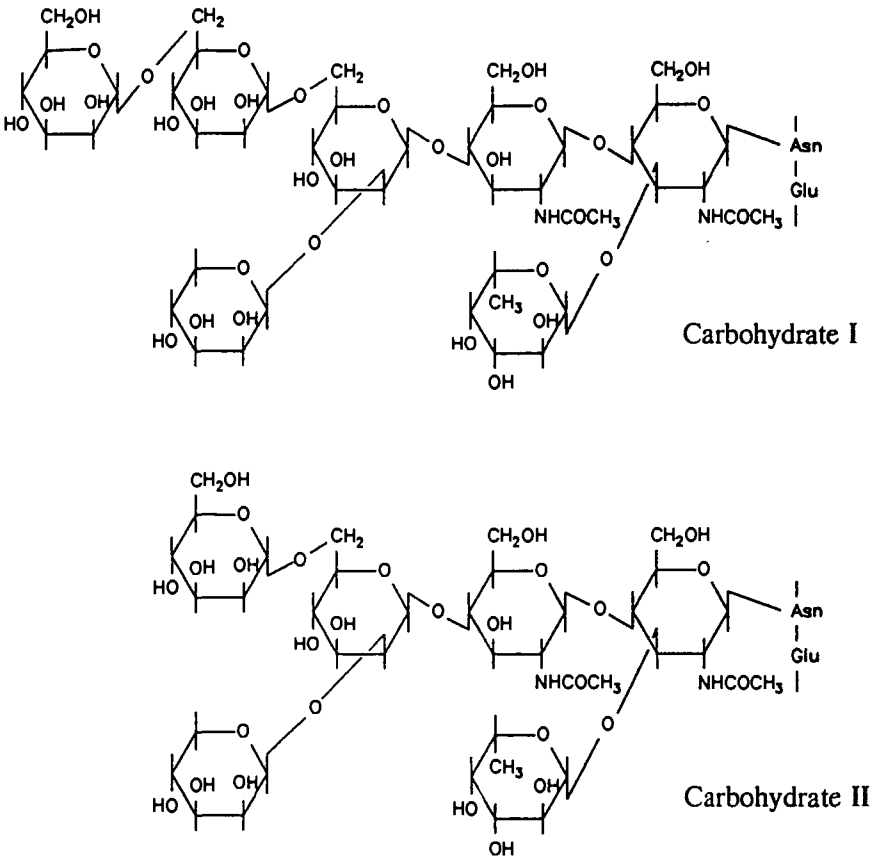

Figure 2 Structures of sugar chains 1 and 2 from stem bromelain. (From Ref. 42.)

oxydative degradation of the carbohydrate residues by sodium periodate did not cause much alteration in enzymic activity toward casein as well as synthetic substrates [52]. The conformation of the *N*-glycan of stem bromelain on the intact glycoprotein was studied by Lommerse et al. [53].

The sequence of stem bromelain contains one sulfhydryl group and three intrapeptide disulfide bonds [45]. The homology between stem bromelain and members of the papain family was first demonstrated by alignment of cyanogen bromide fragments of stem bromelain with the sequence of papain [48,54] and by studying the immunological cross-reactions between different cysteine proteinases [55–57].

Stem bromelain is a more basic protein than papain with a pI of 9.45, while fruit bromelain is an acidic protein. Optical rotatory dispersion measurements [58] and circular dichroism [59] showed that stem bromelain is stable up to pH 10.3. Stem bromelain exhibits a conformational response to increasing alkalinity through at least three different stages. In the first stage, from pH 7 to 10, the protein conformation is stable. In this pH range, 9 out of 19 Tyr hydroxyl groups are accessible to the solvent. In the second stage, from pH 10 to 12, a change in conformation is indicated by changes in viscosity and optical rotatory dispersion parameters. Above pH 12 a large change either in viscosity or in sedimentation coefficient can be observed [59]. With BAEE or Na-benzoyl-L-arginine amide (BAA) as a substrate, the pH profile of $K_{m(app)}/k_{cat}$ shows a wide plateau in the range from pH 5 to pH 8 [42]. This is in general agreement with the pH profile of kinetic parameters for papain and ficin.

For soluble and immobilized bromelain, temperature increase is accompanied by a decrease in residual enzyme activity. A more complex form of denaturation occurs with the immobilized enzyme, which may involve a two-phase process. Immobilization offers more resistance to denaturation at the higher temperature of 60°C where the second phase is prolonged by a factor of three [60]. Differential scanning calorimetry experiments showed that bromelain is an exceptional protease among the cysteine proteases, illustrated by the fact that its thermal denaturation is consistent with an irreversible two-state model [61]. Also, the far UV circular dichroism spectrum of bromelain differs from those of papain and chymopapain and therefore represents a third spectral class within the cysteine proteinase family [62].

B. Catalytic Properties

Bromelain is a member of the cysteine proteinases, a group of enzymes that depends on the thiol group of a cysteine residue for its enzymic activity. Evidence was given for an S-acyl-enzyme intermediate by observing an absorption band having a maximal intensity at 316 nm after admixture of bromelain and methyl thionohippurate. In the acylation step, a controversial (i.e., either a proton-donating or a proton-withdrawing) function is assigned to the imidazole group of the unique histidyl residue, which is spatially very close to the sulfhydryl group [63]. The active-site Cys is situated within a 5 Å distance from an imidazole of histidine as shown by cross-linking studies with 1,3-dibromoacetone [64]. From the results of photo-oxidation experiments, an entirely different mechanism has been proposed in which the histidyl residue may not have intimate electronic interaction with the substrate molecule during catalysis [65]. Photosensitized oxidation of stem bromelain in the presence of methylene blue results in partial loss of the enzymic activity even when the essential sulfhydryl group is protected

from the oxidation. The photo-oxidation involves histidyl, methionyl, and tryptophanyl residues [65,66].

The active site Cys of stem bromelain is located at position 26 (Fig. 1). The alignment of the amino acid sequence of bromelain with those of other cysteine proteases papain, cathepsin H, L, and B, papaya proteinase III, actinidin, and human calpain shows that the enzyme is a member of the papain superfamily [45]. The sequence along the active-site Cys (Cys-Gly-Ala-Cys-Trp-Ala-Phe in papain) is highly conserved by the different members of the cysteine proteinases. The active-site His (His^{159} of papain) is retained in all sequences. The sequence alignment of papain and bromelain (Fig. 1) gives indications for the observed differences between these two proteases. The substitution of Ser^{22} (papain) by Pro, and of the highly conserved Ser^{25} by Ala, may affect the orientation of the catalytic Cys^{26}. In the N-terminal part, the carboxyamide $N^{\varepsilon 2}$ atom of Gln^{20} is conserved. This residue stabilizes the tetrahedral intermediate by taking part in the formation of the oxyanion hole [67]. Val^{158} (papain) is substituted by Leu in bromelain. Val^{158} is responsible for the substrate specificity of papain [67]. This is in accord with differences in specificity found between papain and stem bromelain [68].

Insolubilization of stem bromelain with carboxymethylcellulose as carrier changes the catalytic behavior of the enzyme against substrates of low and high molecular weight [69–71]. The tyrosyl residues in stem bromelain, which are in an exposed state and hence readily accessible to the solvent, can be acetylated with *N*-acetylimidazole at pH 7.5 or nitrated with tetranitromethane at pH 8.0 without change in catalytic activities [72].

C. Inhibition Profile

Being a cysteine proteinase, bromelain is sensitive to inactivation by thiol-blocking reagents and is activated by reagents expected to regenerate thiol groups from disulphides. Bromelain is, however, unusual among plant cysteine proteinases in its weak inhibition by cystatin and by other inhibitors of the cystatin superfamily including L-kininogen. Rowan et al. [21] showed that the K_i of bromelain with chicken cystatin is 3.6×10^{-5} M, at least seven orders of magnitude weaker than that of papain ($<5 \times 10^{-12}$ M). The partial inhibition of bromelain by cystatins is, according to Barrett et al., caused by contamination of commercial preparations of bromelain with other cysteine proteinases that are inhibited by cystatins [73]. Indications for the reasons for the weak binding by cystatin were proposed by Ritonja et al.: Asn^{176} (papain sequence, Fig. 1) has been found in all papain homologs sequenced previously, with the exception of the calpains and of bromelain, which are unaffected by most cystatins. The highly conserved Ser^{177} is mutated to Lys. In papain, Asn^{176} is hydrogen-bonded to

His^{160} and orients it for its catalytic function [54]. The putative catalytic thiolate-imidazolium pair at the active site of bromelain is thus, by comparison of the amino acid sequence of bromelain with other cysteine proteinases, likely to have a different conformation from that in the cysteine proteinases that are tightly inhibited by cystatin [45]. Bromelain also distinguishes itself from other cysteine proteinases by its slow inhibition by the irreversible inhibitor of cysteine proteinases E-64 [(*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl)-amido(4-guanido)butane]. The inhibition of bromelain by E-64 is characterized by a second rate order constant of 512 $M^{-1}s^{-1}$ [27], in contrast with that of 638,000 $M^{-1}s^{-1}$ for papain and 250,000 $M^{-1}s^{-1}$ for ananain [21]. Stem bromelain is reversibly inhibited by thiamine disulfide derivatives (eg., thiamine propyldisulfide, thiamine tetrahydrofurfuryldisulfide [58], tetrathionate [15]) and organic mercurials [35]. 2,2′-Dipyridyldisulfide [38,74] reacts stoichiometrically with the single thiol group in bromelain.

It is irreversibly inhibited by 1-3 dibromoacetone, *N*-(4-dimethylamino-3-5dinitrophenyl)maleimide, *N*-ethylmaleimide, and iodoacetic acid. The chymotrypsin inhibitors *N*-tosyl-L-phenylalanyl-chloromethane (TPCK) and *N*-tosyl-L-lysylchloromethane (TLCK) alkylate the essential SH group [38]. Di-isopropylfluorophosphate (DFP) alkylates the Tyr residues of bromelain but does not react with its catalytic SH. It causes no inhibition of the caseinolytic activity, but the catalytic efficiency against BAEE is enhanced [75].

Human plasma inhibits the hydrolysis of casein by stem bromelain [76]. Stem bromelain is inhibited by α_2-macroglobulin, but not by α_1-antitrypsin [38,77].

A potent cysteine proteinase inhibitor PCPI 8.3 was isolated from potato tubers. The inhibitor has a broad inhibitor spectrum, including the bromelain enzymes, which are not inhibited by cystatin. PCPI shows a K_i value of 190 nM for stem bromelain, 33 nM for fruit bromelain, 0.06 nM for ananain, and 3.3 nM for papain [78]. It is proposed that the differences of inhibitory spectrum between PCPI and the cystatins may be those of distinct superfamilies of cysteine proteinase inhibitors.

V. PHYSIOLOGICAL ACTION OF BROMELAIN

A. Absorption of Bromelain

One of the most important questions to be answered is whether it is possible to absorb intact bromelain in the gastrointestinal tract following oral administration. Smyth et al. reported the absorption of dye-labeled bromelain [79] and I^{131}-labeled bromelain [80]. Izaka et al. [81] used I^{125}-labeled bromelain to

demonstrate the absorption of the intact molecule and degradation products. By means of immunological techniques, gel permeation chromatography, and electrofocusing, they showed that 9.2% of the administered radioactivity was absorbed and that 21% of this radioactivity corresponded with intact absorbed high molecular weight bromelain.

Oral administration of bromelain to rats resulted in an increase in proteolytic activity in blood [82]. Seifert et al. [83] administered radioactive bromelain via the duodenum to rats. They proved that 40% of the radioactively tagged bromelain was absorbed in its high molecular weight form in blood and lymph. The enzyme was identified using antibromelain antiserum raised in rabbits. They claimed that a proteolytic enzyme of about 28,000 daltons passed into the blood and there exerts its physiological action [84].

White et al. [85] also administered I^{125}-labeled bromelain orally to rats. At various times blood was drawn and the total radioactivity, the trichloroacetic acid precipitable I^{125} compounds, and the molecular weight profiles of the labeled proteins were determined. They found a maximum absorption in the blood, corresponding to 270 ng/mL bromelain, one hour after bromelain administration. Approximately 40% of the radioactivity in plasma could be precipitated by 10% trichloroacetic acid. Electrophoretic analysis showed one major peak of radioactivity in plasma, with a molecular weight of 26,000–30,000 daltons. This maximum peak contained 0.003% of the administered dose of bromelain per mL plasma.

The absorption of orally administered bromelain in humans was proven by means of measurement of proteolytic activities using the fluorogenic substrate Z-Arg-coumarin and by immunoprecipitation followed by electrophoresis and immunodetection. The results showed that orally administered bromelain is found in plasma of human volunteers at low, but clearly measurable, concentrations as a full-size proteolytically active protein. The bromelain circulating in plasma appears to be partially bound to certain plasma proteins, two of which were identified as α_2-macroglobulin and α_1-antitrypsin [86].

B. Bromelain and Inflammation

1. Antiedema Action of Bromelain

Bromelain has been used as an anti-inflammatory agent and to heal edema from the time it became available as a drug [2,4–7]. More recent experimental studies demonstrate that bromelain preparations are effective in the prevention and/or inhibition of experimentally induced edema in various animals (for a review, see Ref. 87).

It was shown that bromelain reduced edema in rats in a dose-dependent fashion. Uhlig [88] administered bromelain by means of a duodenal probe to rats with traumatic edema. Compared to a control group, there was a significant reduction of the edema as well as in volume at the time of regression. Pirotta et al. [89] showed dose-dependent antiedema activity in carrageenan-induced footpad edema of rats. After bromelain treatment they observed a statistically significant reduction in edema. The antiedema response was proportional to the logarithm of the administered dose of bromelain. Yamaguchi et al. [90] also found a dose-dependent action of orally administered bromelain on carrageenan-induced edema. A mixed preparation of trypsin and bromelain showed a more pronounced inhibition of edema formation than bromelain alone, suggesting a possible synergism between the two enzymes.

Paw edema induced in rats by kaolin and carrageenan was potentiated by ramipril, an angiotensin-converting enzyme inhibitor, but pretreatment of rats with 10 mg/kg bromelain, intravenous administered, completely prevented this potentiation by ramipril [91]. Rat pleurisy induced by intrapleural injection of kaolin was suppressed by bromelain [92].

2. Anti-Inflammatory Action of Bromelain

Bromelain has found application in a wide variety of inflammatory conditions. These include thrombophlebitis, skeletal muscle injuries, hematomas, oral inflammations, diabetic ulcers, and athletic injuries [2,5,88,93].

Its mechanism of action, however, is not completely elucidated. Kamakura et al. [94] studied the effects of stem bromelain on the plasma kallikrein system, bradykinin levels and plasma exudation at the inflammatory site in rats with a kaolin-induced inflammation of an air pouch. Bromelain caused a dose-dependent decrease of bradykinin levels (measured with the method of Minami et al. [95]) at the inflammatory site and a parallel decrease of the prekallikrein levels in sera [88]. Plasma exudation was also reduced dose-dependently. Bradykinin-degrading activity in sera was elevated after bromelain treatment, but not in the pouch fluid. The authors conclude that bromelain inhibits plasma exudation through inhibition of the bradykinin generation at the inflammatory site via depletion of the plasma kallikrein system. Bromelain also shows a dose-dependent analgesic effect in concanavalin A–injected paws of 5.6 mg/kg i.v.), considered to be due to decrease of high molecular weight kininogen [96].

Uchida and Katori [97] measured the levels of high and low molecular weight kininogen and of prekallikrein in rat plasma after a single intravenous injection of 10 mg/kg bromelain. They found that high molecular weight kininogen and prekallikrein levels were reduced, but low molecular weight kininogen was not. After induction of rat pleurisy by intrapleural injection of

carrageenan, the same results were obtained in the exudate. Fujiyoshi et al. [98] obtained equivalent results in mice. Intraperitoneal injection of kaolin produced a writhing reaction, which did not take place when mice were pretreated with bromelain (10 mg/kg i.v.). Plasma was obtained from mice with or without pretreatment and the kinin release was measured. The bromelain-treated mouse plasma released no detectable amount of kinin, whereas plasma without treatment released about 300 ng/ml of kinin. Furthermore, the prekallikrein activation by kaolin was completely diminished in the presence of soybean trypsin inhibitor.

It was also shown that bromelain, as well as other proteinases, can cleave low molecular weight kininogen from human plasma [99]. All the identified cleavage sites cluster in two stretches of 11–12 residues of kininogen heavy chain, representing the primary attack sites for proteinases. As a result of proteolysis, single domains of 20,000 daltons and combined domains of 40,000 daltons are released from the kininogen heavy chain. These release products can function as cysteine proteinase inhibitors. From the presence of kininogen heavy chain domains in plasma samples under pathological conditions, the authors conclude that cleavage of the proteinase sensitive regions might occur in vivo.

The anti-inflammatory action of bromelain preparations is the result of different mechanisms that act simultaneously. First there is, as described above, the depletion of the plasma kallikrein system, inhibiting the generation of bradykinin, a known chemical mediator of inflammation. Second, there is a negative action on the prostaglandin pathway. Vellini et al. [100] found that bromelain decreased the prostaglandin E_2 levels at the inflammatory site in rats, indicating interference with the arachidonic acid metabolism. Vance [2,101] reported the selective inhibition of prostaglandin E_2, which stimulates inflammation, by bromelain. Taussig [2,101] suggested that bromelain favors the action of the anti-inflammatory prostaglandins (prostaglandin E_1) and prostacyclin (PGI_2), which become predominant, resulting in the stimulation of intracellular cyclic-3,5-adenosine monophosphate (cAMP). As a result it is believed that bromelain is able to reverse the inflammatory reaction.

C. Tumor Growth Inhibition

Gérard [102] was one of the first investigators to use high doses of bromelain alone or in combination with other chemotherapeutic agents on cancer patients. There are, however, few controlled studies concerning the beneficial effect of bromelain on various tumors. In vitro studies on three cell lines—Lewis lung carcinoma, YC lymphoma, and MCA-1 ascitic tumor—showed that bromelain preparations caused a dose-dependent growth retardation of these tumors [103]. Inactivation of the proteolytic activity had no influence on the antitumor activity, whereas peroxidase activity was necessary for tumor growth retardation. On the

in pharmaceutical preparations is pharmacologically not a homogeneous substance but a mixture of different constituents (see above).

In medicine, bromelain is effective in the treatment of inflammation and edema in a wide variety of tissues [2,4–7,103,108], to cure digestive disorders by means of replacement therapy [109], and in cardiovascular and circulatory disorders [95], and it may be important in protecting the small intestine from microbial colonization and disease by modifying intestinal receptor sites and reducing the binding properties of the intestinal mucosa [110]. The antiaggregating effect on platelets together with the proteolytic and fibrinolytic activity in blood are especially responsible for the observed effect in cardiology [32,111].

An interesting application is the use of bromelain in the debridement of third degree burns [112], in the treatment of blunt injuries to the musculoskeletal system [113], and in frostbite eschar removal [114]. It is also used as a potentiating agent in antibiotic therapy [115]. In laboratory medicine it is used extensively in blood group serology [116] and in immunology research [117].

Industrial use of bromelain is rather limited. Its most important use is in the baking industry [118].

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other hand, Maurer et al. [104] reported that bromelain in vitro incubated with myeloid leukemic cells (M1-T22 cell line) stimulated these cells to express lysozyme activity, phagocytosis, and monocytic morphology in a dose-dependent manner, concomitantly with a markedly reduced cell growth. Inhibition of the proteolytic activity of bromelain (heating, inhibition with antipain or leupeptin) gave a 50% reduction of the lysozyme activity, whereas the reduction of cell growth was practically abolished. In human promyelocytic leukemia (HL-60 cells) bromelain induced in vitro monocytic morphology and inhibited cell growth in a dose-dependent manner.

Batkin et al. [105] demonstrated the antimetastatic potential of orally administered bromelain with or without proteolytic and anticoagulant activity on mice. The mice had Lewis lung tumor cells subcutaneously implanted. Although the number of animals used in this study was rather small, the total number of lung metastases was significantly less in animals treated with bromelain than in the control group regardless of the enzymatic activity.

Adamietz et al. [106] treated Ehrlich ascites tumor cells, cultured in vitro, with bromelain. When exposure to bromelain was started at the time of cell plating, there was a temporary block of DNA synthesis, followed by a growth acceleration 48 hours later. Tumor cells already adapted to the substratum and subsequently treated with bromelain showed a similar effect. Interruption of the exposure to bromelain reversed the stimulation effect.

The studies mentioned above indicate that bromelain has a certain cytotoxic potential. It remains an open question whether the observed antineoplastic effects of bromelain preparations reside in the proteolytic enzyme or in some or more other components of the mixture. Before the work of Maurer et al. [104] and of Batkin et al. [105], the antitumor activity of bromelain was explained primarily by its fibrinolytic and platelet aggregation inhibitory activity, which is believed to interfere with the fibrin and coagulation features of tumor cells [2]. More recently, Desser and Rehberger [107] demonstrated that bromelain stimulates the production of alpha tumor necrosis factor in human peripheral blood mononuclear cell cultures in a time-dependent manner. Immunomodulation, especially the release of cytokines, is believed to be responsible for the possible therapeutic potential of bromelain. However, further experimental evidence is necessary, first, to prove the antineoplastic action of the proteolytic enzyme, and second, to demonstrate that bromelain in vivo is a valuable therapeutic agent in humans.

D. Applications of Bromelain

Bromelain preparations are widely used in medicine, as laboratory reagents, and to a lesser extent in industry. It is important to keep in mind that bromelain used

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Hyaluronidase

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I. INTRODUCTION

In 1929 Duran-Reynals [1] described the spreading effect of testicular extracts. Following intradermal injection of a vaccinal virus mixed with the extract, an important increase in injury to the skin was observed. Further investigations using India ink provided additional evidence of the existence of a "spreading factor," which promoted the diffusion of substances through the skin.

In 1936 Meyer and coworkers [1] described an enzyme in autolysates of certain pneumococci that hydrolyzed hyaluronan. In 1940 Chain and Duthie [1] showed that the testicular extract described by Duran-Reynals also hydrolyzed hyaluronan. Similar activity was shown in virulent and avirulent strains of type I pneumococci, in group C hemolytic streptococci, in the skin of rabbits, in the venom of many poisonous snakes, and in bee venom.

The term hyaluronidase was introduced in 1940 to denote enzymes that degrade hyaluronan. Hyaluronidase has been used by many authors as a synonym for "spreading factors." This is only partly correct, as all hyaluronidases act as spreading factors, but not all spreading factors are hyaluronidases [1].

II. HYALURONAN AND GLYCOSAMINOGLYCANS

Hyaluronan is a negatively charged high molecular weight linear polysaccharide built from repeating disaccharide units with the structure — [D-glucuronic acid-β(1→3)-*N*-acetyl D-glucosamine-β(1→4)]$_n$ —. Hyaluronan is ubiquitously distributed in the extracellular space of higher animals and plays a structural role in

cartilage and other tissues. Hyaluronan chains are bound specifically to cartilage proteoglycans, the bond being stabilized by link proteins [2]. Proteoglycans are macromolecules that consist of a central core of protein to which are attached some highly charged glycosaminoglycan chains. These molecules are distributed throughout the extracellular matrices of connective tissues. Interactions between hyaluronan and proteoglycans form multimolecular aggregates. These increase the hydrated volume of the proteoglycan and facilitate entrapment within the collagen network of the extracellular matrix [3]. (The physiological and cell biological functions of hyaluronan [2] and proteoglycans [4] have been extensively studied.)

Glycosaminoglycans found in connective tissues are divided in five main classes [3]. Their chemical structures are given in Figure 1.

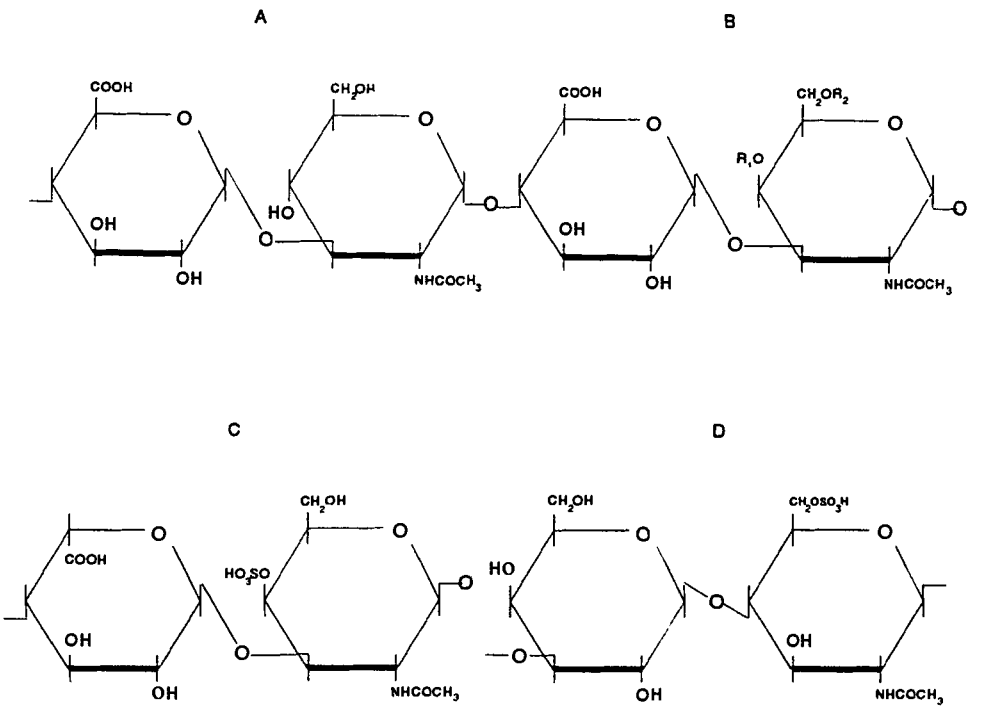

Figure 1 Molecular structure of (A) hyaluronan, (B) R_1 = H, R_2 = H: chondroitin; $R_1 = SO_3H$, R_2 = H: chondroitin-4-sulfate; R_1 = H, $R_2 = SO_3H$: chondroitin-6-sulfate, (C) dermatan sulfate, and (D) keratan sulfate.

A. Chondroitin and Chondroitin Sulfate

Chondroitin is an unbranched heteropolymer consisting of repeating disaccharides of D-glucuronic acid and *N*-acetyl D-galactosamine: — [D-glucuronic acid-β(1→3)-*N*-acetyl D-galactosamine-β(1→4)]$_n$ —. The galactosamine residue may be O-sulfated at either C-4 (chondroitin-4-sulfate or chondroitin sulfate A) or C-6 (chondroitin-6-sulfate or chondroitin sulfate C).

B. Dermatan Sulfate (Chondroitin Sulfate B)

Dermatan sulfate is an epimer of chondroitin sulfate. The uronic acid residue in the repeat disaccharide is found as L-iduronic acid: — [L-iduronate-α(1→3)-*N*-acetyl D-galactosamine-β(1→4)]$_n$ —. The galactosamine residue is sulfated at C-4.

C. Keratan Sulfate

In keratan sulfate the repeating disaccharide consists of galactose linked to *N*-acetyl glucosamine: — [D-galactose-β(1→4)-*N*-acetyl D-glucosamine-β(1→3)]$_n$ —. The glucosamine residue is sulfated at C-6.

D. Heparin

Heparin has a more complex structure, containing both iduronic and glucuronic acid residues, and, in addition, many glucosamine residues can be *N*-sulfated. Heparin is particularly highly, though variably, sulfated. The repeating unit consists of D-glucuronic or L-iduronic acid linked to D-glucosamine: — [hexuronic acid α/β-(1→4)-D-glucosamine-β(1→4)]$_n$ —. The amino group may be either sulfated or acetylated, and C-6 on the glucosamine residue is generally sulfated. The iduronic acid residues may also be sulfated at C-2.

E. Heparan Sulfate

Heparan sulfate is very closely related to heparin, but it contains higher proportions of glucuronic acid and is less O-sulfated and more *N*-acetylated.

III. DISTRIBUTION AND CLASSIFICATION

Hyaluronidases are divided into three main classes according to their mechanism of action on hyaluronan [5].

A. Hyaluronoglucosaminidase (Hyaluronate 4-Glucanohydrolase, E.C. 3.2.1.35)

This type of hyaluronidase randomly hydrolyzes the 1,4-linkages between the *N*-acetyl β-D-glucosamine and the D-glucuronate residues in hyaluronan. The enzyme is subdivided according to its source.

1. Testicular Hyaluronidase

Commercial preparations usually contain this type of hyaluronidase. The enzyme is found in testicular tissue of most mammals and is located in the acrosomal cap of spermatozoa [6]. Testicular hyaluronidase degrades hyaluronan, chondroitin, chondroitin-4- and -6-sulfate to oligosaccharides, mainly tetrasaccharides [1]. Partial degradation of dermatan sulfate has been described [7]. Testicular hyaluronidase had a broad pH range of activity [5].

Testicular hyaluronidase has been shown to possess transglycosylation properties. Incubation with hexasaccharide digests of hyaluronan leads to the production of higher oligomers [8]. Even incubation with mixtures of hyaluronan and chondroitin sulfate leads to oligosaccharides containing mixtures of the repeating units of both polysaccharides [9]. The full mechanism of the transglycosylation reaction has been studied by Highsmith et al. [10].

2. Tissue or Lysosomal Hyaluronidase

Bollet et al. [11] demonstrated the presence of hyaluronidase activity in various mammalian tissues. They showed that this type of hyaluronidase differed from the testicular type concerning pH optimum and pH range of activity. Subsequent studies revealed that the enzyme was present in the lysosomal fraction of the tissues [12]. The liver is an especially rich source [13]. Degradation of hyaluronan leads to the same end products as testicular hyaluronidase [11]. Lysosomal hyaluronidase from rat liver degrades chondroitin-4- and -6-sulfate, but not dermatan sulfate, desulfated dermatan sulfate, heparan sulfate, keratan sulfate, or heparin [14]. Lysosomal hyaluronidase has an acid pH optimum and a narrow pH range of activity [14]. This difference in pH profile of activity has commonly been used to differentiate between testicular and lysosomal hyaluronidase. A similar acid-active hyaluronidase is present in human serum [15].

Lysosomal hyaluronidase possesses transglycosylation properties using octasaccharides as a substrate, rather than hexasaccharides like testicular hyaluronidase [14,16].

3. Venom Hyaluronidase

Animal venoms usually possess hyaluronidase activity [17]. The enzymatic properties, including hyaluronidase, of snake venoms have been extensively studied by Tan et al. [18]. Snake hyaluronidase acts on hyaluronan, chondroitin, and chondroitin-4- and -6-sulfate, producing various oligosaccharides, mainly tetrasaccharides [1].

Hyaluronidase from bee venom has about the same substrate specificity as snake hyaluronidase [19]. Venom of social wasps was found to contain high levels of hyaluronidase activity, whereas venom from ants contains low levels of activity [20]. Lizard venom contains a hyaluronidase that acts almost specific on hyaluronan, i.e., it has no activity toward chondroitin-6-sulfate, dermatan sulfate, or heparin and only weak activity toward chondroitin-4-sulfate [21].

A hyaluronidase has been purified and characterized from stonefish (*Synanceja horrida*) venom. It acts specifically on hyaluronan, producing tetra-, hexa-, octa-, and decasaccharides, but does not act on chondroitin sulfate or dermatan sulfate [22,23].

High amounts of hyaluronidase are present in scorpion venom [17]. The enzyme is also present in the venom of tarantula (*Eurypelma californicum*) [24] and in the venom of the poisonous brown recluse spider (*Loxosceles reclusa*) [25].

Table 1 presents a survey of hyaluronidase activity in the venom of several animal species. Transglycosylation properties have been observed with hyaluronidase from snake venom (*Crotalus terrificus*) [8], but not with hyaluronidase from bee venom [19].

B. Hyaluronoglucuronidase (Hyaluronate 3-Glycanohydrolase, E.C. 3.2.1.36)

Hyaluronoglucuronidase hydrolyzes the 1,3-linkages between β-glucuronate and *N*-acetyl D-glucosamine residues in hyaluronan. This type of hyaluronidase can be readily extracted from the heads of leeches. The medicinal leech (*Hirudo medicinalis*) is commonly used as the source of this enzyme, but a tropical Asian species of leech (*Sanguisoga granulosa*) can serve as an alternative source [26]. Leech hyaluronidase degrades hyaluronan, producing a tetrasaccharide with glucuronic acid at the reducing end. It shows no activity toward chondroitin and chondroitin-4- or -6-sulfate [27]. Leech hyaluronidase possess no transglycosylation properties [8].

A novel hyaluronan-degrading enzyme has been isolated, purified, and characterized from Antarctic krill (*Euphausia superba*) [28]. Preliminary investigations show that the enzyme acts as an endo-β-glucuronidase.

Table 1 Hyaluronidase Activities of Some Animal Venoms

Species	Activity[a]	Ref.
Snake		
Micrurus corallinus	83	18
Micrurus nigrocinctus	272	18
Bungarus multicinctus	186	18
Crotalus basilicus	17	66
Echis coloratus	68	17
Oxyuranus scutellatus	88	17
Lizard		
Heloderma horridum	530/857	17/66
Heloderma suspectum	532/857	17/66
Scorpion		
Centruroides limpidus limpidus	94	66
Pandinus imperator	1956	17
Heterometrus spinnifer	1451	17
Spider		
Segestria florentina	34	17
Wasp and bee		
Vespula pensylvanica	301	17
Vespa basalis	59	17
Apis mellifera	283–674	17
Ant		
Pogonomyrmex barbatus	166	17
Paraponera clavata	17	17
Pogonomyrmex rugosus	9	66

[a]National Formulary Units/mg venom.

C. Hyaluronate Lyase (E.C. 4.2.2.1)

This type of enzyme is commonly obtained from bacterial sources like pneumococci, staphylococci, streptococci, clostridia [29], *Flavobacterium,* and *Proteus vulgaris* [30], and has been demonstrated in *Treponema pallidum* [31]. Bacterial hyaluronidases degrade hyaluronan to a disaccharide containing a Δ-4,5-unsaturated uronic acid by eliminating 1 mole of water from the uronic acid portion of the repeating unit of hyaluronan [29]. The full mechanism of this type of reaction has been elucidated by Ludowieg et al. [32].

Hyaluronidase from *Flavobacterium* and *Proteus vulgaris* has been shown to hydrolyze chondroitin-4- and -6-sulfate and to possess weak activity toward dermatan sulfate [30]. Hyaluronidase from *Streptomyces hyalurolyticus*

hydrolyzes hyaluronan to unsaturated tetra- and hexasaccharides, but does not act on chondroitin sulfates, chondroitin, heparin, and keratan sulfate [33].

Hyaluronidase activity has been demonstrated in streptococcal phages [34] and in infective hookworm (*Ancylostoma*) larvae [35]. The latter has been shown to possess specific activity toward hyaluronan.

IV. BIOLOGICAL FUNCTIONS

A. Spreading and Virulence Factor

Hyaluronan is present in the extracellular space and plays a structural role in cartilage and other tissues. At physiological concentrations the hyaluronan molecules entangle and form a random network of chains, excluding or retarding the diffusion of larger molecules through this network [2]. Hyaluronidase acts on the extracellular matrix macromolecules, altering its composition and structure [36]. The enzyme enhances the absorption of drugs [37], promotes the spreading of venoms [21], and the virulence of bacteria has often been associated with the production of hyaluronidase [35,38,39].

B. Fertilization

Mammalian oocytes are surrounded by several layers of cells embedded in an extracellular matrix. This matrix has been shown to contain protein and hyaluronan [40]. The fertilizing spermatozoon must pass through it to reach the egg. In 1942 McClean and Rowlands demonstrated the possible role of hyaluronidase in dispersing and digesting the layers surrounding the oocyte [41]. Subsequent studies have shown that hyaluronidase is not essential, but is a helpful tool in assisting the passage of the individual sperm toward the oocyte [40].

C. Cancer

Tumors are often enriched in hyaluronan. Cancer cells produce high levels of hyaluronan or stimulate other cells to produce the polysaccharide. This hyaluronan halo protects the cell from cytotoxic lymphocytes [2]. Other investigators suggested that hyaluronan promotes the locomotion of cells by opening migration paths [2]. An important aspect of this function is tumor invasion. West et al. demonstrated that hyaluronidase digests of hyaluronan have angiogenic (formation of new blood vessels) properties [42]. All these facts suggest that hyaluronan can play an important role in tumor invasion. The biological role of hyaluronidase in cancer seems dubious. Fiszer-Szafarz and Szafarz demonstrated

that the lysosomes of hepatomas did not contain any hyaluronidase activity, whereas hyaluronidase is present in the lysosomes of normal liver cells [43]. Hyaluronidase activity in hepatomas was significantly increased in the extracellular space, changing the properties of the tumor cell surface and the pericellular region, thus facilitating invasiveness and metastasis. On the other hand, De Mayer and De Mayer-Guignard showed that enhanced levels of hyaluronidase activity in the serum of mice provided a better protection against the development of tumors [44]. The enhanced levels of hyaluronidase should lead to a more pronounced action on the hyaluronan coatings surrounding the cancer cells, making them more accessible to cytotoxic lymphocytes.

D. Differentiation and Modeling of Tissues

Many investigations have shown that hyaluronidase activity is found in tissues capable of undergoing rapid resorption or remodeling [45,46]. During mitosis lysosomes lose important enzyme activities (including hyaluronidase) [47]. This process is most likely under hormonal control of the thyroid [48]. Hyaluronan has been shown to be involved in the process of cell proliferation, cell recognition, and locomotion [2]. In many developing and regenerating systems the change in hyaluronan production is correlated with an inverse change in hyaluronidase activity, and high levels of hyaluronan synthesis are subsequently followed by increased levels of hyaluronidase activity [49].

V. EXTRACTION AND PURIFICATION

A. Extraction

Testicular hyaluronidase is extracted from testicular tissue or semen. The crude material is homogenized and centrifuged. The supernatant is subjected to direct precipitation with $(NH_4)_2SO_4$ [50] or to extraction with Triton-X and hyamine, followed by precipitation with ethanol [51] or $(NH_4)_2SO_4$ [52]. Harrison, however, reported a simple extraction of hyaluronidase from ram semen after centrifugation by washing the pellet in the presence of NaCl (0.3 M) and Mega 9 (5 mg/mL), a nonionic detergent [53]. Lysosomal hyaluronidase from several tissues [54,55] and bacterial hyaluronidase [56] has been extracted using similar procedures.

B. Purification

Further purification of the crude enzyme preparations obtained after extraction often involves multiple chromatographic steps including ion-exchange, gel

filtration, and affinity chromatography [53]. The complete extraction and purification, as employed by Harrison [53], is shown in Figure 2.

Lyon and Phelps [57] have evaluated various glycan-substituted AH-Sepharose gels to be used for affinity chromatography. Srivastava and Farooqui reported the elution of hyaluronidase from heparin-Sepharose columns using heparin or hyaluronan solutions as eluent [58]. Alternatively, affinity chromatography of hyaluronidase has been performed using concavalin A–Sepharose [58] or Matrex gel phenyl boronate [59] columns.

VI. ENZYMATIC PROPERTIES

A. pH Profile

A difference in pH activity profile has historically been one of the principal distinctions between different types of hyaluronidase, especially testicular and

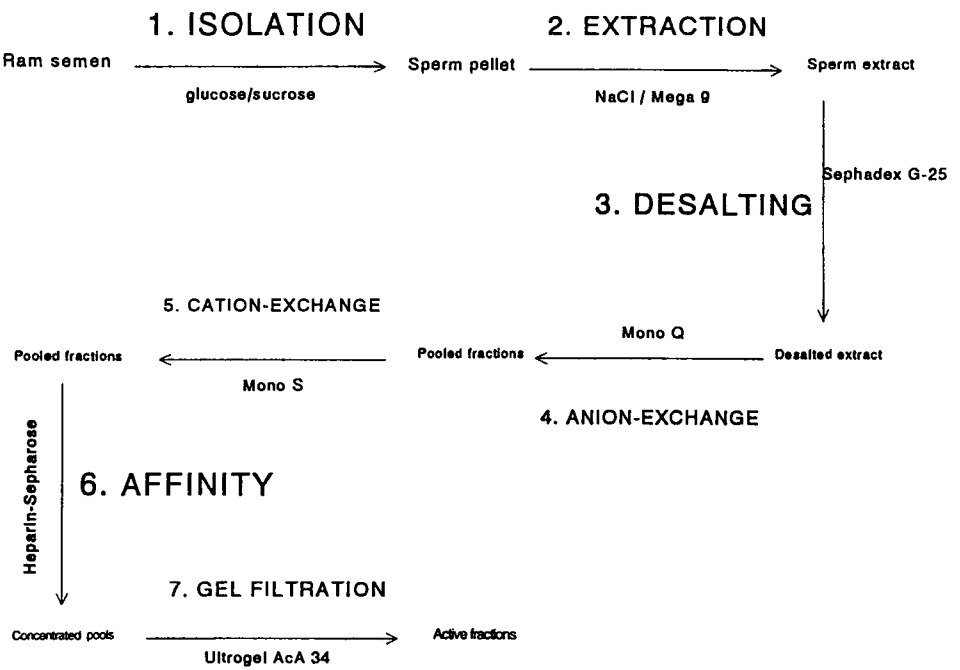

Figure 2 Extraction and purification of hyaluronidase from ram semen. (From Ref. 53.)

lysosomal hyaluronidases. Testicular hyaluronidase has a broad pH range of activity (3.5–8), with an optimum at about 3.8. It shows no sign of activity below pH 2.6 [50]. Lysosomal hyaluronidase has about the same pH optimum, but shows more activity in acidic media and no activity above pH 4.8 [54]. The pH optimum and profile depend strongly on the ionic strength and the composition of the buffer used [60].

B. Ionic Strength

Hyaluronidase has an absolute requirement for cations, as dialyzed preparations show no activity toward dialyzed substrates. K^+, Na^+, Ca^{2+}, Mg^{2+}, and Mn^{2+} all have stimulating properties toward hyaluronidase. The monovalent cations show the strongest stimulating effects and act over a broader range of concentration than divalent cations [50]. Testicular, serum, and lysosomal hyaluronidase seem to have different sensitivities toward NaCl [54,61]. A noncompetitive mechanism was proposed to explain the activation of hyaluronidase by Na^+ [59,62].

C. Molecular Weight and Polymorphism

Using chromatographic, electrophoretic, or immunological methods, several investigations have shown that testicular, lysosomal, or bacterial hyaluronidase can exist in multiple forms [52,63–66]. Harrison [63] showed that the various hyaluronidase forms isolated from ram semen were members of an oligomeric series, formed by intermolecular disulfide cross-linking of two monomeric species, designated α (M_r 89,600) and β (M_r 81,200). Gupta and Goldberg [52] investigated the immunological specificity of mouse testicular hyaluronidase. They demonstrated that the enzyme is immunologically cell and species specific. Reaction with the antibodies resulted in one precipitation line, indicating that the enzyme is immunologically homogeneous and that the different bands obtained after electrophoresis represent multiple forms of the enzyme.

The molecular weight of hyaluronidases can be readily obtained using sodium dodecyl sulfate polyacrylamide gel electrophoresis or by gel filtration chromatography. The molecular weight values for testicular hyaluronidase range from about 60,000 to 90,000 [50,63]. Lysosomal hyaluronidase appears to have a molecular weight within this range [54,55,59]. Using SDS-PAGE with hyaluronan-containing gels, Cevallos et al. [66] investigated the polymorphism and the molecular weight of hyaluronidase from several venom sources. All of the hyaluronidases from the invertebrate venoms had molecular weights below 50,000 and showed only one component, while those from vertebrate venoms weighed more than 60,000 and in many cases contained more than one form.

D. Molecular Structure

Hyaluronidases are generally glycoproteins. This has been confirmed for testicular [67], *Staphylococcus aureus* [68], lysosomal [59], lizard venom [21], and stonefish venom [22] hyaluronidases. The amino acid compositions of testicular [67], *Staphylococcus aureus* [68], lizard venom [21], and stone-fish venom [22] hyaluronidases have been determined. Of the latter type, the N-terminal sequence has been derived. The hyaluronidase gene of *Streptococcus pyogenes* bacteriophage H4489A has been expressed in *Escherichia coli* and the full nucleotide sequence has been derived. This made possible a mapping of the full amino acid sequence of the enzyme [69].

Lace et al. proved that testicular hyaluronidase is a glycoprotein of the high-mannose type [70]. They removed the carbohydrate moiety of the enzyme using endoglycosidase F and showed that this did not alter the activity and the antigenicity of the enzyme.

Inhibition experiments using *p*-chloromercuribenzoate and iodoacetate confirmed that thiol groups are not involved in the reaction catalyzed by testicular hyaluronidase [58]. It has been reported that lysine residues are not involved in the catalytic reaction or the binding of the substrate to the enzyme [62]. Inhibition experiments using butane-2,3-dione demonstrated that at least one arginine residue is essential for enzymatic activity. This arginine residue is probably involved in the binding of the substrate to the enzyme [62]. Similar results have been reported with leech hyaluronidase [71]. Shanidze et al. suggested that histidine residues are essential for the activity of *Staphylococcus aureus* hyaluronidase [72].

E. Thermal Stability

Solutions of crude preparations of hyaluronidase can be stored frozen for several months without losing important amounts of activity [26,50,73]. Purified hyaluronidase rapidly loses all activity when stored frozen unless stabilizing agents like detergents or high concentrations of salts are added [63]. At neutral pH, solutions of hyaluronidase can be stored at room temperature for several days [50]. The temperature profile of activity of the enzyme shows a sharp decline at a temperature of about 50°C. Hyaluronidase is rapidly inactivated when stored at 55–60°C [14,50,52], but it can be kept at 40°C for at least one hour without losing activity [30,50].

F. Stimulation and Inhibition

1. Stimulation

Hyaluronidase is activated by mono- or divalent cations and by polycations. Doak and Zahler [74] demonstrated stimulating effects of bovine serum albumin,

histone, protamine sulfate, spermine, spermidine, and hyamine. The stimulating properties were dependent on the pH of the assay and are thought to evolve from an interaction between the polycationic substance and hyaluronan. The products obtained from ultraviolet radiation of 8-methoxypsoralen in the presence of polyamines, especially spermine and spermidine, were shown to possess stimulating effects on hyaluronidase [75]. It is thought that these photoproducts may play an important role in the onset of 8-methoxypsoralen–induced photosensitivity. Kakegawa et al. showed that compound 48/80 (a polycondensate of *N*-methyl-*p*-methoxyphenethylamine and formaldehyde) activated hyaluronidase concentration dependently, even in the absence of metal salts [76]. Compound 48/80 and calcium, both strong activators of hyaluronidase, are closely associated with the degranulation process of mast cells, which is a common mechanism in type I allergy. Therefore, inhibitors of hyaluronidase are often investigated as potential antiallergic agents. Kakegawa et al. pointed to the fact that it is preferable to use calcium ions as activating agents when the inhibition of hyaluronidase is examined [76].

2. Inhibition

Several studies have shown that hyaluronidase is inhibited by Fe^{3+}, Fe^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} salts [26,51,58,77,78]. Substrate analogs like chondroitin sulfate B, desulfated chondroitin sulfate B, dermatan sulfate, keratan sulfate, heparitin sulfate, and heparin were shown to be competitive inhibitors of hyaluronidase [14,54].

Flavonoids are well known as inhibitors of hyaluronidase. In 1950 Rodney et al. reported the effect of several flavonoids on hyaluronidase using in vitro and in vivo methods [79]. Kuppusamy et al. investigated the structure-activity relationship of the flavonoids as inhibitors of testicular hyaluronidase [80] and hyaluronidase from snake, scorpion, and bee venom [81]. They showed that flavones, flavanols, and chalcones possess the general ability to inhibit hyaluronidase. Kinetic investigations showed that these compounds acted as competitive inhibitors [80]. Several hyaluronidase inhibitors were shown to be able to delay the action of snake venom when injected into mice [81]. One cannot rule out the possible interaction of the flavonoids with other components of the venom, as the inhibitory effects of flavonoids are not specific to hyaluronidase [79]. Figure 3 shows the inhibitory capacities of some flavonoids on testicular and several venom hyaluronidases.

Inhibitors of hyaluronidase have a potential application as antiallergic agents. The pathology of type I allergy (which produces symptoms like asthma and hay fever, has been explained in terms of the histamine-releasing reaction and the degranulation of mast cells. It is thought that this degranulation is induced by

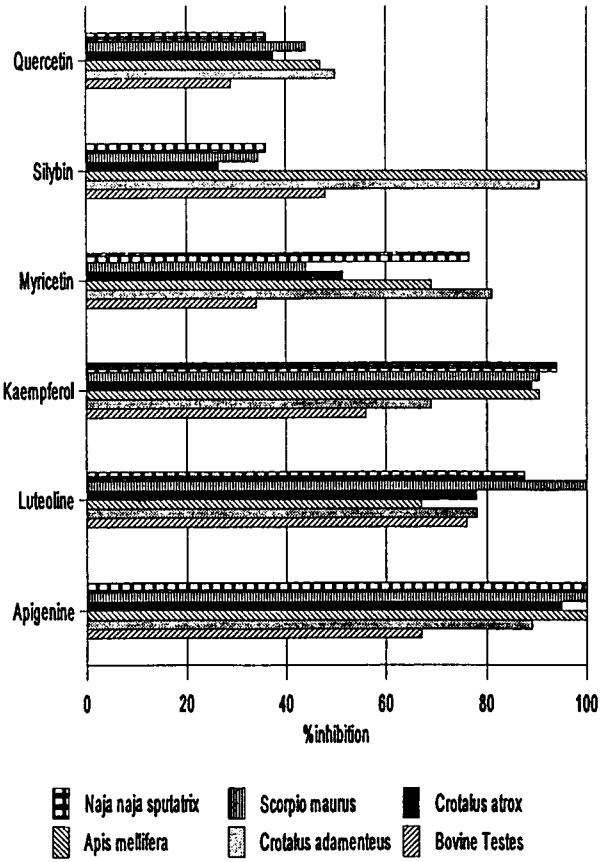

Figure 3 Inhibition properties of some flavonoids (250 mM) on hyaluronidase from several venom sources and from bovine testes. (From Refs. 80,81.)

a flow of calcium ions into the cells, thus activating certain enzyme reactions. Compound 48/80 is known to release histamine, inducing an extensive degranulation of the mast cells. Calcium and compound 48/80 are known to activate hyaluronidase, and therefore Kakegawa et al. investigated the inhibitory effects of disodium cromoglycate and tranilast on hyaluronidase [76]. Both components are used in practice as antiallergic agents and are thought to inhibit the degranulation of the mast cells. Disodium cromoglycate was shown to inhibit the activation of hyaluronidase and activated hyaluronidase when NaCl, $CaCl_2$, or compound

48/80 were used as activators [76]. Tranilast strongly inhibited the activation of hyaluronidase and activated hyaluronidase when $CaCl_2$ or compound 48/80 were used as activating agents. The activation of hyaluronidase was only weakly inhibited when NaCl was added. Moreover, tranilast enhanced the activity of NaCl-activated hyaluronidase [76]. Similar results on the inhibition of the activation of hyaluronidase and the release of histamine from mast cells were reported with photoirradiated products of tranilast [82], tannins [83], hydrangenol derivatives [84], and some natural products [85]. Inhibitory effects on activated hyaluronidase and the histamine release from mast cells were described with pectic substances [86,87]. Pectins are polysaccharides built from D-galacturonic acid, and it has been shown that the inhibitory activity is related to the D-galacturonic acid contents [86]. These substances are competitive inhibitors of hyaluronidase and are postulated to possess antiallergic properties [86]. Inhibitors of hyaluronidase with antiallergic properties were also found in tea extracts [88]. Inhibition of hyaluronidase was also reported with stilbene and dihydrostilbene derivatives [89].

As hyaluronidase plays an important role in fertilization, several investigations dealt with the possible use of hyaluronidase inhibitors as contraceptive agents. During the 1950s flavonoids like phosphorylated hesperidin were evaluated with mixed results [90,91]. Compounds like phosphorylated hesperidin [92,93], PS_{53} (a condensed polymer of formaldehyde and hydroguinone sulphonic acid) [92], tetradecylsodium sulfate [94,95], and sodium aurothiomalate (myocrisin) [93,96] were shown to be hyaluronidase inhibitors in vitro and to possess antifertility activity when administered intravaginally. Marketed anti-inflammatory agents like phenylbutazone, oxyphenbutazone, and fenoprofen were shown to possess vaginal contraceptive properties, though their mechanism of action could be due to an inactivation of cyclooxygenase, which may play a role in the sperm acrosome reaction during fertilization [93]. In vitro studies have shown that fenoprofen possesses hyaluronidase inhibitory properties [97]. It has been reported that human serum contains a nonspecific inhibitor of hyaluronidase, though its nature and possible role in vivo is still poorly established [98].

VII. THERAPEUTIC USE

A. Myocardial Infarction

The first reports on the beneficial effects of hyaluronidase in the treatment of acute myocardial infarction were published by de Oliveira et al. [99]. Hyaluronidase was administered intravenously to dogs in which infarction was

induced experimentally as well as to six patients, all suffering from an acute myocardial infarction. The results were very promising and initiated further clinical investigations. MacLean et al., using rats, obtained positive effects on the myocardial infarct size following the administration of hyaluronidase [100]. Promising results were obtained by Nilsson et al., who administered several intravenous doses of hyaluronidase to rats 2 hours after the onset of the infarction [101]. A reduction of the infarct size was observed by Repa et al. using an intravenous infusion of hyaluronidase 2 hours after the induction of an infarction in dogs [102]. Despite these positive results, small- or large-scale clinical trials on humans failed to prove a significant effect of the administration of hyaluronidase upon the size of the myocardial injury after an infarction [103–105]. These less than clear results for the beneficial effects of hyaluronidase could be due to the rapid removal of the enzyme from the bloodstream upon intravenous administration [106]. Flint et al. observed a trend toward a reduced mortality among a group of patients admitted to treatment within 6 hours of infarction [104]. It appears that therapeutic administration of hyaluronidase should appear within the first few hours of coronary artery occlusion.

De Oliveira et al. explained the effects of hyaluronidase in terms of a reduction of the water content of the ischemic myocardium [107]. Hyaluronidase would reduce the edema during the acute phase following coronary occlusion and thereby reduce the injury. Intramyocardial edema occurs after the occlusion of coronary arteries, resulting in an increased water content of the myocardial tissues. Hyaluronidase produces a faster diffusion of fluids and thereby reduces edema in the damaged area. To examine the effects of hyaluronidase on infarction, Waldenström et al. [108] investigated the accumulation of hyaluronan and water during the development of infarction. Both levels increased progressively for several days. By its water-binding capacity, interstitial accumulation of hyaluronan would contribute to the interstitial edema in infarcted myocardial tissue [108]. These findings could explain the effects of hyaluronidase observed in animal studies. Szalvy et al. investigated the reduction of the cardiac lymph during a myocardial infarction [109]. They showed that hyaluronidase sustained the lymphatic drainage of the myocardium following infarction, thereby reducing the destruction of myocardial tissues. Sustained lymphatic drainage could ease the removal of toxic substances such as lysosomes from the ischemic myocardium. Sunnergren and Rovetto investigated the effect of hyaluronidase on coronary microvascular permeability in the heart [110]. During an infarction the coronary outflow is decreased and the water content of the tissues increased. Hyaluronidase prevented the decrease in coronary outflow and prevented an increase in coronary resistance. By enhancing capillary fluid reabsorption and the lymph flow, hyaluronidase reduces the water content of the tissues.

B. Cancer Therapy

As described above, cancer cells produce or induce other cells to produce hyaluronan. This halo of hyaluronan protects the cells against macrophages or leukocytes and shields them from cytostatics. Several studies have dealt with the addition of hyaluronidase to chemotherapy in order to degrade the hyaluronan coating and to render the cells more accessible to the cytostatic drugs.

Kohno et al., using multicellular tumor spheroids, showed that the addition of hyaluronidase to doxorubicin increased the penetration and cell-killing effect of the drug [111]. This increase depended on the time of exposure and the origin of the tumor cell. In vitro and in vivo studies showed that the combination of hyaluronidase with adriamycin could enhance the antineoplastic activity of adriamycin without increasing the toxic side effects [112]. Höbarth et al. used an intravesicular instillation of hyaluronidase and mitomycin C to treat bladder cancer [113]. By breaking down the protective hyaluronan halo around urothelical cells, diffusion of the drug into the cells was improved, resulting in a reduced recurrence rate. Carcinoma of the head and the neck were treated with polychemotherapy (vindesine, cis-platin), hyaluronidase, and radiation [114]. This treatment was found very effective and well tolerable and occasionally complete remissions were observed. Czejka et al. showed that hyaluronidase decreased the systemic concentration of 5-fluorouracil and thus enhanced the tissue penetration of the drug [115]. In an in vivo pilot study Baumgartner and Baumgartner treated several types of cancer with a combination therapy of hyaluronidase and cytostatics [116]. In many cases long lasting remissions were observed and an improvement in the general condition was noticed. Analogous investigations have been reviewed by Baumgartner et al. [117].

Despite all these positive results, an in vitro study performed by Scheithauer et al. to investigate the combination of hyaluronidase with four cytostatics (doxorubicin, methotrexate, cis-platin and vinblastine) against six cell lines led to disappointing results [118]. However, it was observed that the combined drug-hyaluronidase effect does not depend on the class of anticancer agent used, and the sensitivity toward the combined effect was heterogeneous among the various tumor cell lines tested.

C. Anesthesia for Optical Surgery

Hyaluronidase has found applications as an additive to the anesthetic agents used for peribulbar anesthesia for vitreoretinal surgery. Combinations of hyaluronidase with bupivacaine [119], lidocaine and epinephrine [120], lignocaine and adrenaline [121], or lidocaine and bupivacaine [122] were judged very

satisfactory for anesthesia and also for akinesia. Furthermore, the addition of hyaluronidase often made ophthalmic anesthesia less painful.

D. Various Applications

The following applications for hyaluronidase have been found:

Hyaluronidase, in combination with triamcinolone and lidocaine, has been employed to treat recurrences of keloids after laser resection [123].

In scalp-reduction surgery, hyaluronidase has been combined with the local anesthetics to facilitate their diffusion, to enhance the anesthesia, and to ease the dissection [124].

Hyaluronidase has been shown to have some therapeutic role in the management of tuberculous spinal arachnoiditis [125].

Subcutaneous injection of hyaluronidase (150 U/mL) may decrease the morbidity associated with intravenous extravasation injuries [126].

Intramuscular injection of hyaluronidase (150 U) decreases and softens hematoma at an angiographic puncture site [127].

Brain abscess formation was studied experimentally in rats, and treatment with different combinations of hyaluronidase, dexamethasone, and antibiotic has been investigated [128]. The results showed that combined therapy with antibiotic and hyaluronidase, started the day before inoculation, averted the formation of brain abscess and the therapy, started after encapsulation, effectively eliminated the organisms. However, the same therapy started at the cerebritis stages caused an increase of cerebritis.

VIII. PHARMACOLOGY AND TOXICITY

The fate of intravenously administered hyaluronidase has been studied in humans and rats using ^{125}I- or biotin-labeled hyaluronidase. The serum half-life in humans was approximately 3.2 minutes. Similar half-life values have been measured in rats. Within 10 minutes of administration the bulk of the enzyme accumulated in the liver (>50%) with measurable quantities in the kidneys and spleen. The presence of three high-mannose type oligosaccharide subunits per polypeptide chain probably accounts for the rapid uptake by the liver. Preadministration of mannans or deglycosylation of the enzyme significantly increased the serum half-life of hyaluronidase [106].

Hyaluronidase has practically no serious side effects, although intravenous administration may be associated with severe allergic reactions. The use of highly purified preparations should reduce the likelihood of allergic complications. A clinical and histological study has investigated the safety of intravitreal

injections of hyaluronidase (10–1500 IU/mL 0.9% saline) in the rabbit eye. No abnormalities were found using 10 IU/mL, but at higher concentrations clinical and histological changes were seen in proportion to the concentration used [129].

IX. ASSAYS

A. Quantitative Assays

In this chapter we describe some methods used to determine the kinetics of the action of hyaluronidase. Table 2 presents a survey of the Michaelis-Menten constants (K_m) of the action of hyaluronidase on hyaluronan and chondroitin sulfate obtained using different methods. These assays usually make use of hyaluronan as a substrate for hyaluronidase. Various sources of hyaluronan are employed, but these substrates have different physicochemical properties (molecular weight, intrinsic viscosity). Payan et al. [130] investigated the action of *Streptomyces* hyaluronidase on hyaluronan from several sources.

1. International Hyaluronidase Standard Preparations

An international standard for hyaluronidase was accepted in 1957 by the World Health Organization (WHO) [131]. This was of great historical value, since hyaluronidase is one of the first biological international standard reference preparations ever made. The International Unit (IU) was and is still defined as the activity of 0.1 mg of the International Standard Preparation. It is recommended that the standard be employed for the assay of hyaluronidase from testicular origin (E.C.3.2.1.35). According to the WHO, the standard should be valid for all methods of comparison, including turbidimetric and viscosimetric assays. The first international WHO standard 1955 hyaluronidase is still available in tablet form with an activity of approximately 200 IU per tablet.

The FIP and the European Pharmacopeia have a new BRP hyaluronidase standard power with an activity of 330 IU/mg. The stability and activity of this preparation is tested regularly by interlaboratory collaborative assays. International standards for the standardization of hyaluronidases of nontesticular origin are not available.

2. Turbidimetric Assays

These methods are based on the formation of insoluble complexes between acid mucopolysaccharides (like hyaluronan or chondroitinsulfate) and quaternary ammonium compounds or bovine serum albumin. The amount of turbidity developed is proportional to the amount of acid mucopolysaccharides present in

Table 2 Michaelis-Menten (K_m) Constants of the Action of Hyaluronidase from Different Sources on Different Substrates

Enzyme source	Substrate	Analytical method	K_m (mg/mL)	Ref.
Bovine testes	Hyaluronan	GPC	0.44	139
Bovine testes	Hyaluronan	Turbidimetry	0.30	22
Bovine testes	Hyaluronan	Light scattering	0.18–0.32	132
Bovine testes	Hyaluronan	Determination of end groups	0.55	54
			0.46	138
Bovine testes	Hyaluronan	Viscosimetry	0.51	136
Bull sperm	Hyaluronan	Determination of end groups	3.7	50
Mouse testes	Hyaluronan	Determination of end groups	1.1	52
Human liver	Hyaluronan	Determination of end groups	0.05	54
Pig liver	Hyaluronan	Determination of end groups	0.64	59
Rat liver	Hyaluronan	Determination of end groups	0.08	14
Stonefish venom	Hyaluronan	Turbidimetry	0.71	22
Bovine testes	Chondroitin-6-sulfate	Viscosimetry	2.67	137
Pig liver	Chondroitin-6-sulfate	Determination of end groups	0.28	59
Pig liver	Chondroitin-4-sulfate	Determination of end groups	0.34	59

the system. No turbidity develops when hyaluronan is depolymerized by hyaluronidase.

The current method for the hyaluronidase assay described in the United States Pharmacopeia (USP) [132] is based on the inability of hydrolyzed potassium hyaluronate to form a complex precipitate with proteins from added serum, reflected in a decreased turbidity of the reaction mixture (measured after 30 min). The method is, from the enzymological point of view, not well defined since it does not actually evaluate the kinetics of the hydrolysis of the substrate. An assay with end-point determination is only valid if the reaction rate does not change during this reaction time. We found that only with the two lowest test concentrations (0.15 and 0.3 IU) was this condition fulfilled, while with the three higher test concentrations the reaction is not linear. Commercially available hyaluronates can be contaminated with chondroitin sulfates. They are more acidic than hyaluronic acid itself and hence can form better protein complexes and influence the turbidity. In a suitability test of the USP [133], the substrate must pass both an inhibitor content test and a turbidity-production test. The assumption is made that

each cleavage by the hyaluronidase produces a non–turbidity-producing unit with molecular weight less than 8000. However, Bailey and Levine [134] have recently proven that initial increases of turbidity are possible. Moreover, they have found that passing the USP suitability test is not sufficient to guarantee a suitable substrate. Moreover, the measured turbidities are dependent on the type of spectrometer used.

3. Viscosimetry

The European Pharmacopoeia and FIP standard viscosimetric method is described in Chapter 15. In the proposed method, both hyaluronidase and hyaluronan are available as well-defined stable standard preparations, eliminating variations in test results due to the absence of a well-defined substrate. With slight modifications the method can be used for the determination of hyaluronidase in serum [137].

The response (a decrease of viscosity) is a direct consequence of the action of the enzyme on its substrate, since the splitting of the glycosidic bonds gives a decrease in the viscosimetric average molecular weight and hydrodynamic volume of the hyaluronan chains and hence a decrease in the intrinsic and relative viscosities. With this method the rate at different concentrations cannot be compared because the initial viscosities are different and rheological measurements do not coincide. To eliminate these problems, a kinetic dilution methodology for the viscosimetric study of the substrate concentration dependence of the action of hyaluronidase was proposed [135,136]. We were able to determine the rate of reaction, expressed as the number of moles of bonds broken per unit of time, from viscosimetric data [136].

4. Gel Permeation Chromatography

Considering the theory of the ad random degradation of a polymer, in the initial stage of the degradation a linear relationship between the reciprocal value of the numeric average molecular weight (M_n) and the time of the reaction is to be expected [139]. The slope of the straight line provides the rate of degradation expressed as the number of moles of bonds broken per unit of time. The numeric average molecular weight and other molecular weight averages are commonly obtained using gel permeation chromatography.

Figure 4 presents some typical chromatograms of hyaluronan after digestion by hyaluronidase. By evaluating the decrease of the other molecular weight averages and by comparing the experimental findings with theoretical considerations [139,141], a clear proof has been given that hyaluronidase degrades hyaluronan in an ad random fashion [140].

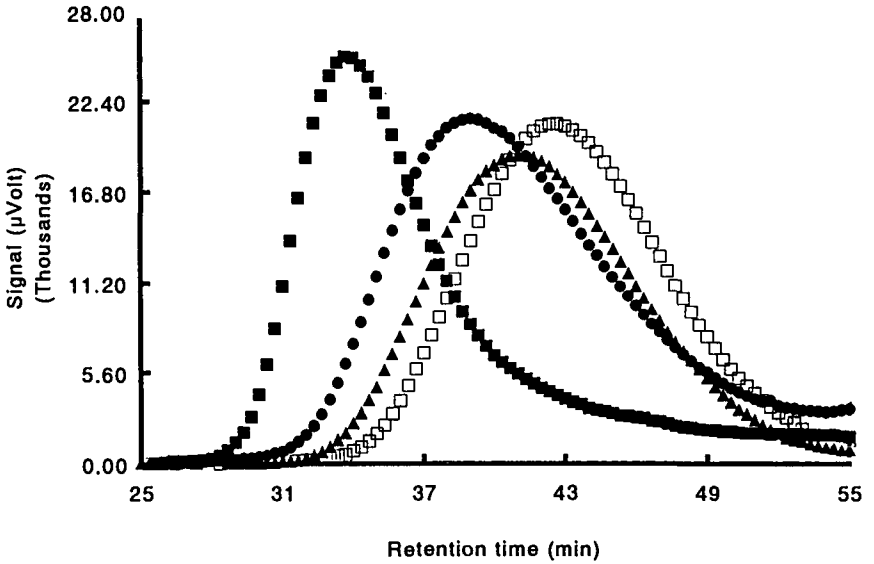

Figure 4 Gel permeation chromatography profiles of hyaluronan (0.3 mg/mL) after 0 (■), 20 (●), 40 (▲), and 60 (□) minutes of treatment with hyaluronidase (0.33 IU/mL). (From Ref. 139.)

5. Dye-Binding Assay

This spectrophotometric method is based on the binding of a carbocyanine dye (1-ethyl-2-[3-(1-ethyl-naphto[1,2*d*]thiazolin-2-ylidene)-2-methyl-propenyl]naphto [1,2*d*]thiazolium bromide, Stains all) to undegraded hyaluronan [34]. The unit of enzyme activity is defined as the amount of enzyme that will produce a 10% decrease in the absorbance of the dye-hyaluronan complex after one hour of incubation.

6. Determination of End Groups

This assay is used to investigate hyaluronidase activities from a variety of sources or to estimate the inhibitory capacities of compounds on the enzyme. The assay is based on the determination of the liberated *N*-acetyl-D-glucosamine end groups from hyaluronan after digestion by hyaluronidase [60]. A comparison has been made between the Morgan-Elson and neocuproine assays and of the influence of inhibitors [138].

7. Light Scattering

This assay is based on the theory of light scattering by random coils undergoing random scission [142]. The authors make several assumptions (e.g., mass independence of the second virial coefficient) and adapt their method to be useful even with simple light scattering instruments, using high scattering angles and a cheap monochromatic light source. In this method the decrease of the molecular weight of the hyaluronan is not determined, although initial reaction velocities can be determined from the decrease of the molecular weight of the sample [143].

8. Fluorimetric Assay

The substrate for this assay consists of hyaluronan with the reducing terminals labeled with the fluorogenic reagent 2-aminopyridine [144]. It should be noted that the reaction products by hyaluronidase also contain oligosaccharides that do not contain the fluorogenic end group. As these products are not detectable by this method, not all scissions occurring in the substrate are detected.

B. Qualitative Assays

These assays are used to confirm the presence of hyaluronidase in a preparation. Quantification can only be achieved by using a standard hyaluronidase preparation of known activity, determined with one of the methods described in the first part of this chapter.

1. Plate Assay

For this assay plates or petri dishes consisting of hyaluronan dispersed in agar and buffered to the appropriate pH are used. Cylindrical holes are punched in the gel and the samples (unknowns or standards) are added. The plates are incubated at 37°C for several hours. After incubation, a solution of a quaternary ammonium compound is poured over the gel, and after reaction the areas of digested hyaluronan become visible as clear rings. This technique is a simple assay for hyaluronidase activity in biological samples and has been used to estimate hyaluronidase activity in hymenoptera venoms [145], in semen, and in seminal plasma [146].

2. Slide Assay

This technique is a histochemical procedure to evaluate the hyaluronidase activity of spermatozoa. Using this technique, Waibel et al. evaluated the inhibition of mouse testicular hyaluronidase by sodium aurothiomalate [147]. Hirayama et al.

[148] used the slide assay to evaluate the hyaluronidase activity of human spermatozoa and the potential inhibition by sodium aurothiomalate.

3. ELISA-Like Assays

These assays make use of probes that bind specifically to hyaluronan, but not to the digestion products after degradation by hyaluronidase. The remaining binding capacity is evaluated using a second probe coupled to an enzymatic reaction. Delpech et al. [61] made use of hyaluronectin, a proteoglycan that binds specifically and reversibly to hyaluronan. The activity is calculated according to the suppression of immune complex binding.

Stern et al. [149] made use of a hyaluronan-binding protein obtained from the tryptic digestion of the proteoglycan core protein of bovine nasal cartilage to detect hyaluronidase activities in urine samples from Wilms' tumor patients. The pathophysiology of this tumor is associated with major alterations in the metabolism of hyaluronan, and it is thought that urinary hyaluronidase can be used as an additional marker.

4. Electrophoretic Assays

These techniques allow one to detect hyaluronidase activities in crude biological samples or extracts from bacterial, animal, or human sources. They make possible a differentiation between the different forms of the enzyme. The assays commonly make use of polyacrylamide gels that contain hyaluronan [64,150].

Most authors apply native polyacrylamide gels without sodium dodecyl sulfate, because this detergent would reduce enzymatic activity. Cevallos et al. [66] used SDS-PAGE to analyze hyaluronidase samples. This technique allows the demonstration of hyaluronidase activity and the determination of the molecular weight of the proteins present in a sample. Steiner and Cruce [151] developed a zymographic assay to evaluate hyaluronidase activities in biological samples.

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9

Pancreatic Enzyme Replacement

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I. PANCREATIC SECRETION

The pancreas secretes a juice having two major components—an alkaline fluid and enzymes—into the duodenum. The two components occur in variable proportions depending on the stimuli. The alkaline fluid component, ranging in volume from 200 to 800 mL/day, has a high concentration of bicarbonate, which neutralizes the gastric contents entering the duodenum and helps to regulate the pH of the intestinal tract. The enzymes of pancreatic juice are synthesized and secreted by the acinar cells, which also secrete a fluid similar in electrolyte composition to an ultrafiltrate of plasma. In intermittent feeders such as humans, dogs, and cats, the volume of fluid secreted is very small and has little effect upon the volume and composition of pancreatic juice flowing in the main pancreatic duct.

Four major enzyme groups are secreted: lipolytic, proteolytic, amylolytic, and nucleic acid splitting enzymes. These pancreatic enzymes, some of which are secreted in multiple forms, possess specificities complementary to the intestinal membrane-bound enzymes (Table 1). Fresh, uncontaminated pancreatic juice is without proteolytic activity because these enzymes are in the form of inactive zymogens. An important fraction of the calcium in pancreatic juice accompanies the enzymes, especially α-amylase. Human pancreatic juice is most close to that of the pig, with high proportions of lipase and α-amylase in comparison with other mammals [1]. Therefore, pig pancreas extract, pancreatin, has up to now been the preferred enzyme source for therapeutic pancreas substitution.

Table 1 Complementary Action of Digestive Enzymes in Intestinal Lumen

Natural substrates	Pancreatic hydrolases	Intestinal brush border hydrolases
Triglycerides	Lipase-colipase (EC 3.1.1.3)	
Phospholipids	Phospholipase A_2 (EC 3.1.1.4)	
Cholesterolesters, esters of vitamins	Carboxylester lipase (EC 3.1.1.1)	
Xenobiotics		
Proteins, large peptides	Trypsin (EC 3.4.21.4)	
	Chymotrypsin (EC 3.4.21.1)	
	Elastase (EC 3.4.21.36)	
Oligopeptides	Carboxypeptidase A (EC 3.4.17.1)	Aminopeptidase A (EC 3.4.11.7)
	Carboxypeptidase B (EC 3.4.17.2)	Aminopeptidase N (EC 3.4.11.2)
		Aminopeptidase P (EC 3.4.11.9)
		Aminopeptidase W (EC 3.4.11.16)
		Dipeptidylpeptidase IV (EC 3.4.14.5)
		Carboxypeptidase P (EC 3.4.17.16)
		Angiotensin-converting enzyme (EC 3.4.15.1)
		Neutral endopeptidase (EC 3.4.24.11)
Starch (amylose, amylopectin)	α-Amylase (EC 3.2.1.1)	
Dextrin, maltotriose, isomaltose, sucrose		Sucrase-isomaltase (EC 3.2.1.10-3.2.1.48)
Amylose, maltotriose, maltose		Maltase-glucoamylase (EC 3.2.1.20)
Lactose		Lactase (β-galactosidase) (EC 3.2.1.108)
Nucleic acids	Ribonuclease (EC 3.1.27.5)	
	Desoxyribonuclease I (EC 3.1.21.1)	
	Desoxyribonuclease II (EC 3.1.22.1)	
Nucleotides, nucleosides		Nucleotidases (EC 3.1.3.31-3.1.3.6)
Organic phosphates		Alkaline phosphatase (EC 3.1.3.1)

II. PANCREATIC LIPOLYTIC ENZYMES

While it was previously thought that the lipase of pancreatic origin, the classical lipase, was responsible for the digestion of most dietary fat, it has become clear in recent years that lipolysis in the gastrointestinal tract is a result of the concerted action of four different types of lipases: gastric lipase, classical pancreatic lipase, pancreatic carboxylester lipase (identical to cholesterol esterase), and phospholipase A_2 (Table 2).

Naturally occurring glycerides are esters of *sn*-glycerol, where *sn* stands for stereospecific numbering according to the IUPAC nomenclature for lipids. The three hydroxyl groups in glycerol are designated as *sn*-1, *sn*-2, and *sn*-3.

An enantiomeric triglyceride has a specific fatty acid esterified to each position. Thus, 1-palmitoyl-2-oleyl-3-linoleoyl-*sn*-glycerol has the formula depicted in Figure 1. Diglycerides can occur in three isomeric forms, for example, 1,3-dipalmitin, 1,2-dipalmitin, and 2,3-dipalmitin, and likewise there are three isomers of a monoglyceride such as monopalmitin: 1-monopalmitin, 2-monopalmitin, or 3-monopalmitin.

Gastric lipase and pancreatic lipase are the two major triacylglyceride ester hydrolases (EC 3.1.1.3) in mammals, but they probably have no effect on the recently introduced synthetic polyglycerol esters used as emulsifiers in food and as dietetic aids (Fig. 1).

The lipases and phospholipase A_2 differ from classic esterases in that their natural substrates are insoluble in water and their activity is maximal only when the enzyme is adsorbed to the oil/water interface. Therefore a special treatment of the enzyme kinetics of these enzymes is imposed. The term substrate concentration becomes different, as only the substrate present in the interface is available for the enzyme. Consequently, the interface itself becomes the substrate. While in homogeneous systems, the enzymatic work space is in three dimensions, and substrate concentrations are expressed in terms of volume, the concentration of insoluble substrates only has meaning when expressed as interface area/volume or, when dealing with two-dimensional kinetics, as moles/area.

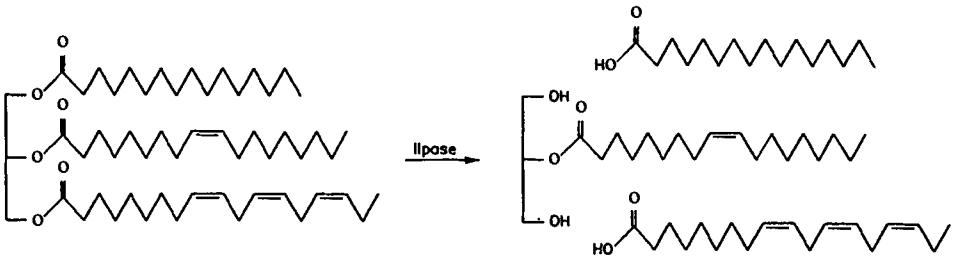
Parameters defining the quality of the interface such as enzyme orientation, substrate packing, hydrophobicity, charge density, and film pressure come into consideration [2–4], and terms characterizing enzyme activities such as V_{max} and K_m have different meanings.

A. Pancreatic Lipase

Lipase constitutes only 1–3% of the protein in mammalian pancreatic juice. This relatively low proportion is compensated for by a very high molecular activity since one enzyme molecule can cleave nearly 7000 ester bonds per second under

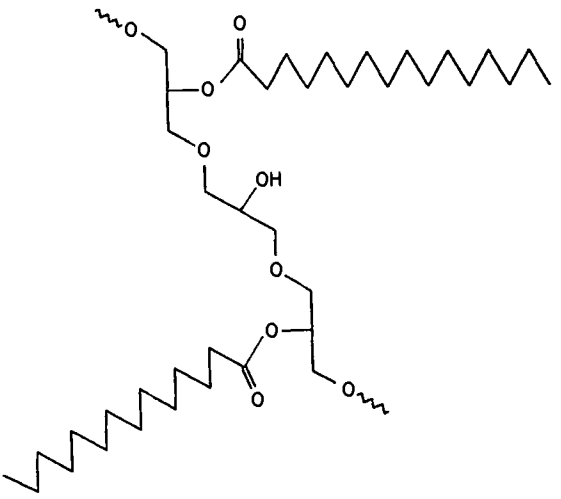
Table 2 Lipolytic Enzymes in the Gastrointestinal Tract

	Stomach	Small intestine		
	Gastric lipase	Pancreatic lipase	Carboxylester lipase Cholesterol esterase	Phospholipase A_2
EC classification		3.1.1.3	3.1.1.1 or 3.1.1.13	3.1.1.4
Tissular origin	Tongue Pharynx Stomach	Exocrine pancreas	Exocrine pancreas (Breast milk)	Exocrine pancreas
Substrate specificity	*sn*-3 position of triglycerides >> *sn*-1, *sn*-2	*sn*-1 and *sn*-3 positions of triglycerides	Cholesterol esters, esters of vitamin A, E xenobiotics, glycerides (low specific activity)	*sn*-2 position of L-α-glycerophospholipids
Essential cofactors		Colipase	Bile salts required for hydrolysis of insoluble substrates	Ca^{2+} ions



1-palmitoyl-2-oleyl-3-linoleoyl-sn-glycerol

(a)



Polyglycerol

(b)

Figure 1 (a) Esters of glycerol with fatty acids can be hydrolyzed by the different types of human lipase with varying specificities, while (b) synthetic esters of polyglycerol with fatty acids are resistant.

optimal conditions. Porcine pancreas, from which lipase was first purified, is one of the richest sources [1].

1. Substrate

Pancreatic lipase has been found to be strictly *sn* 1,3–specific, that is the primary fatty acid esters of the triglyceride. Although the two primary hydroxyl groups on

the glycerol molecule are sterically distinct, the enzyme is unable to differentiate between them. The enzyme has a higher activity toward tri- than diglycerides, while 2-monoglycerides are resistant to hydrolysis [5].

The formation of monoglycerides may be of physiological importance, not only because of their role as emulsifying agents but also because their uptake leads to a saving of energy in the subsequent process of resynthesis of triglycerides. Fatty acid chain length has only a minor influence on pancreatic lipase activity, as lengths from 3 to 20 carbons all are hydrolyzed at rates within ±50% of that of oleic acid [6]. Highly unsaturated marine oils were found to be poor substrates of pancreatic lipase, and fatty acids desaturated in the Δ2-Δ6 positions are resistant to hydrolysis by pancreatic lipase [7].

2. Molecular Properties

Pancreatic lipase, a single chain glycoprotein (48 kDa) [8], is found to be a serine hydrolase, although it is not inhibited by classical serine hydrolase inhibitors like diisopropyl fluorophosphate (DFP) and phenyl methyl sulfonyl fluoride (PMSF). pH titration and photo-oxidation studies have demonstrated that a histidine residue is required for enzymatic activity. X-ray crystallography [9], site-directed mutation [10], and inhibition studies with tetrahydrolipstatin [11] indicate that the Ser 152 in human pancreas lipase represents the nucleophilic residue essential for catalysis and is part of the Asp 176-His 263-Ser 152 triad, which is chemically analogous to, but structurally different from, that in the serine proteases. However, this site is covered by a surface loop and therefore not directly accessible to the substrate, which is in agreement with the very low activity noticed on soluble substrates. An interfacial activation is required to induce a reorientation of the surface loop, flap, or lid [9,12,13] and the creation of an oxyanion hole for catalytic action. The structure of the flap contains hydrophobic residues, which may upon binding to the interface penetrate into the substrate micelle. However, in some of the prokaryotic lipases the Asp residue in the active site is mutated to a Glu [14]. The serine residue from the active triad is part of a conserved Gly-X-Ser-X-Gly sequence found in all lipases sequenced up to now, including the evolutionarily unrelated prokaryotic lipases [15–17].

As illustrated in Figure 2, human pancreatic lipase consists of two domains. The larger N-terminal domain, a mixed α,β-structure, comprises residues 1–335 and contains the catalytic triad and a calcium-binding site. The smaller C-terminal domain is formed by two layers of antiparallel β-strands and presents a binding site for colipase.

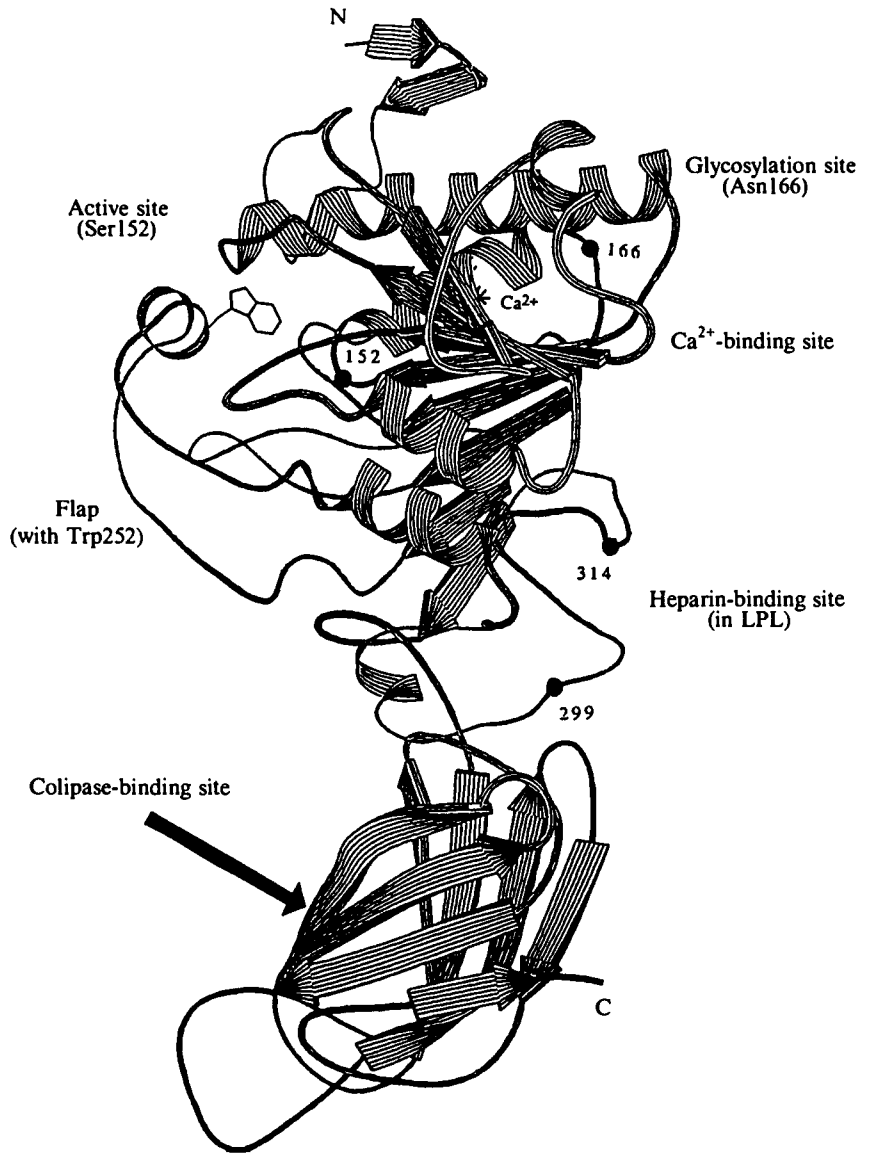


Figure 2 Structure of human pancreatic lipase with indication of the active site buried beneath a mobile Trp 252 surface loop (flap) in order to give access to the substrate and the colipase-binding site faced in the same direction as the active site. The enzyme is glycosylated at Asn 166 (porcine), stabilized at the surface segment by a Ca^{2+}-binding site (partly hidden, marked by an asterisk), and can be anchored by means of the heparin-binding site to heparan sulfate on cell surfaces. (From Ref. 8.)

3. Colipase

Colipase is a small (12 kDa) pancreatic protein displaying a remarkable stability toward pH fluctuations [18] (Fig. 3), heat, and organic solvents, which facilitates its purification from fresh pancreas or a side fraction of insulin production. Colipase consists essentially of three fingers and is topologically comparable to the snake toxins. The tips of the fingers contain the majority of the hydrophobic amino acids and presumably form the interfacial binding site [13]. In the presence of colipase, the inhibitory effect of even high bile salt concentrations becomes neutralized [19]. It is a quite specific phenomenon as mammalian colipases are only able to reactivate mammalian lipases and not microbial lipases [20]. On the other hand, the inhibitory influence of bile salts on the interface and lipase activity lacks specificity, and not only mammalian lipase but many microbial lipases too are sensitive to a variety of amphiphilic compounds such as bile salts [21]. Furthermore bile salts and high concentrations of calcium and other cations can induce a shift of the pH optimum of pancreatic lipase and pK_a of long-chain fatty acids from 9 to below 6.5 [22]. This lowering of the pH optimum by bile salts is attributed to their inhibition of binding of the enzyme to triacylglycerol particles at alkaline pH. Albumin, the principal biological carrier of long-chain fatty acids, enhances lipase activity at low concentrations (10^{-7} M) and inhibits it at high concentrations ($>10^{-6}$ M) [23,24].

The influence of bile salts in physiological proportions is illustrated in Figure 4, where the curve is the result of opposite effects of colipase and bile salts. Maximum activities are reached in the range of 5–13 mM bile salts, a concentration also present in the duodenum during fat digestion. In the FIP method for pancreatin lipase, about 10 mM FIP controlled sodium taurocholate is used.

Pancreatic lipase is rapidly inactivated at pH values less than 4 [25] (Figs. 3 and 5), which has important consequences in the development of galenic formulations for pancreatic enzyme replacement.

4. Assay Systems

Because of the existence of the interfering carboxylester lipase activity (Table 2) in the pancreas, dissolved substrates are not adequate for the quantitative assay of pancreas lipase. A satisfactory technique is the measurement of the fatty acids, liberated from a long-chain triacylglycerol emulsion, in a recording pH-stat. In special circumstances, tributyrylglycerol emulsions without a stabilizer and releasing freely soluble products are preferred. Indeed, preparation of a long-chain triacylglycerol emulsion requires stabilization by an emulsifier or by

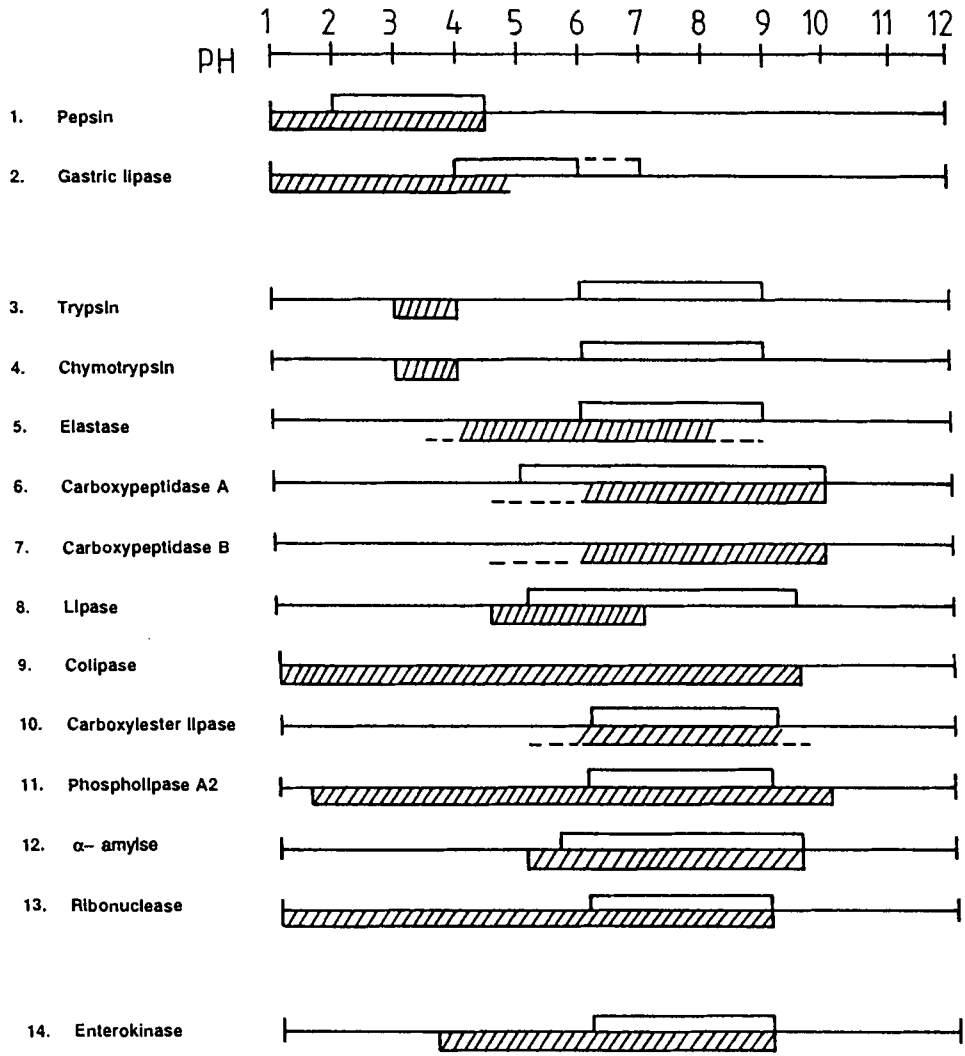


Figure 3 Ranges of activities (upperside) and stability (underside) in function of pH of the major digestive enzymes present in human gastrointestinal tract. Non-well-documented zones are represented by a broken line. The ranges indicated are only approximate, as they can be influenced by many factors and reaction conditions, e.g., the range of activity of gastric lipase shifts from pH 4–6 (long-chain triglycerides) to pH 4–7 (short-chain triglycerides).

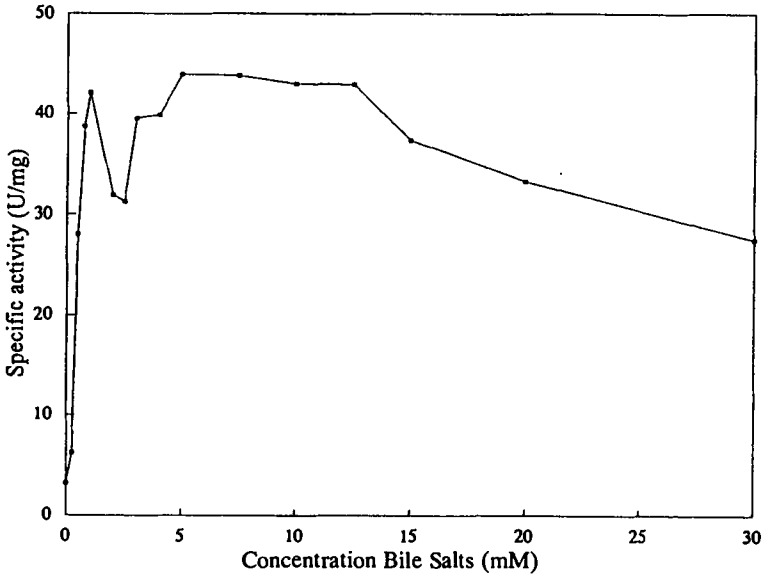


Figure 4 Effect of increasing concentration of bile salts in physiological proportions on the activity of pancreatin lipase (FIP standard LS7). Reaction conditions are neutralized olive oil, 100 g/L; hydroxypropyl methylcellulose, 11 g/L; calcium, 10 mM; NaCl, 100 mM; and a mixture of bile salts (29.8% glycocholate, 24.5% glycochenodesoxycholate, 11.9% glycodeoxycholate, 12.6% taurocholate, 13.6% taurochenodesoxycholate, 7.6% taurodeoxycholate).

another means. Arabic gum is a frequently used stabilizer, but the presence of considerable amounts of bivalent cations, especially calcium, might influence enzyme activity measurements. The polymer hydroxypropyl methyl cellulose (HPMC) seems to combine several advantages: there is no contamination by bivalent cations and stable emulsions can be prepared because a suitable level of viscosity is obtained using high concentrations of the polymer without interfering with the enzyme [26]. Since lipolytic activity depends on the total surface area of the oil droplets, prepared emulsions should be well characterized and stable. Fine emulsions can be obtained with a high-speed blender, but because of the inclusion of air bubbles and the lack of homogeneity of the oil droplet sizes, phase separation rapidly occurs. Using ultrasonication, far more stable emulsions are produced: droplets are more homogeneous in size and oil droplet size distributions can easily be obtained with a supermicron particle sizer (Fig. 6). Lipase activity can also be measured by a variety of routine methods using colorimetric

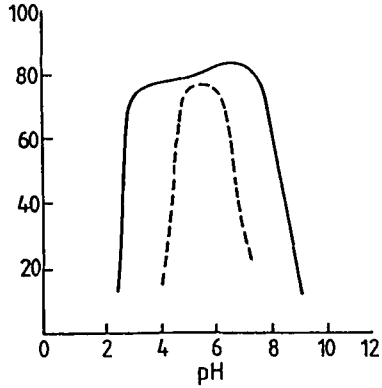


Figure 5 Stability in function of pH of pancreatin lipase (broken line) compared with a fungal lipase (*Rhizopus javanicus*) (solid line). The pancreatin lipase was incubated for 3 hours at 37°C and the fungal lipase for 4 hours. After the preincubation period the activity was measured using the FIP procedure.

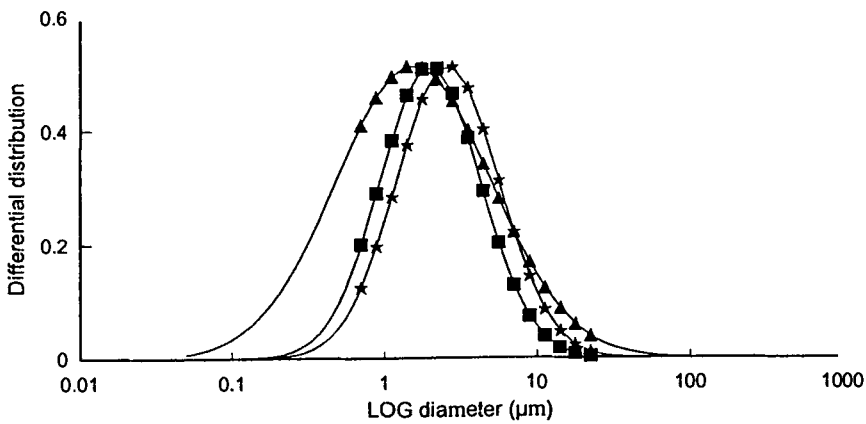


Figure 6 Oil droplet size distribution of an olive oil emulsion, stabilized with hydroxypropyl methylcellulose, after different emulsification procedures: blender (triangles), ultrasonic probe (squares), and ultrasonic homogenizer (stars). Theoretical distributions were calculated from Coulter Counter measurements using a software program, assuming spherical particles.

and ultraviolet absorbance assays, decrease in turbidity of triolein emulsions, and immunoassay techniques [27]; however, a sufficient amount of colipase should be present to attain maximal reaction rates. Pancreatin preparations fulfill these conditions as they contain large amounts of colipase [28].

Recently, we adapted the Ortho Clinical Diagnostics lipase slide test [29], a dry chemistry test system used in clinical biochemistry to measure the lipase activity in human serum for the determination of the lipase activity in pancreatic extracts. The substrate, 1-oleyl-2,3-diacetylglycerol, and the ancillary reagents are fixed in a multilayered film (Fig. 7). Upon addition of a lipase solution, the insoluble substrate becomes emulsified.

Solutions of a pancreatin FIP standard preparation in physiological saline were diluted 1:1 with a solution of 8% BSA, 0.9% NaCl and measured with this fast, completely automated method. The Ortho method yielded an excellent correlation with the FIP pH-stat reference method: Ortho U/L = 0.968 FIP U/L + 4.232 ($r = 0.999$). The recoveries in the low (294 FIP U/L), medium (440 FIP U/L), and high (734 FIP U/L) activity ranges were 101.1, 99.9, and 96.5%, respectively; the intra-assay CVs were 3.0, 1.9, and 1.6% ($n = 5$). The detection limit, determined by a two-step dilution method and defined as the highest dilution that can be distinguished from the zero solution with 95% confidence, was 3.2 Ortho U/L.

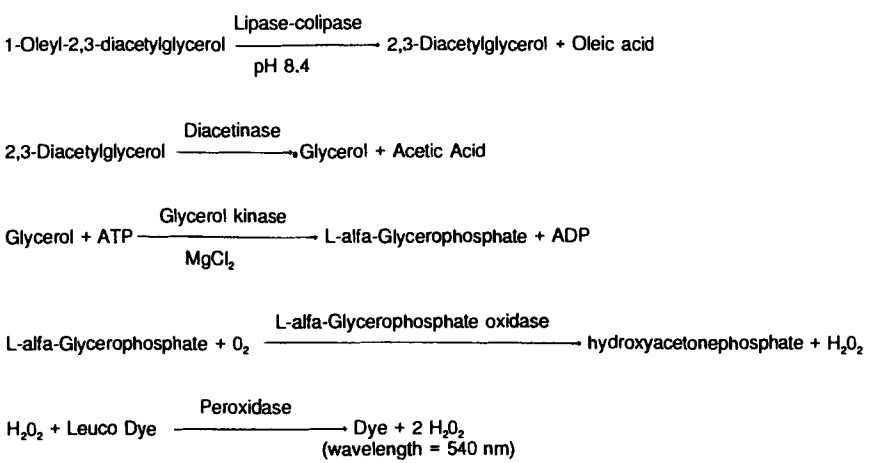


Figure 7 Reaction proceedings in the Ortho Clinical Diagnostics lipase slide test. 1-Oleyl-2,3-diacetylglycerol serves as an artificial substrate for lipase.

B. Pancreatic Phospholipase A_2

The best explored mammalian phospholipase A_2 is the type I 14 kDa pancreatic enzyme released as a proenzyme. The 14 kDa phospholipases have been classified utilizing evolutionary relationships into type I (in pancreas juice), type II (in platelets, synovial fluid), and type III (in bee venom). Platelets and macrophages contain intracellularly a structurally different 85 kDa phospholipase type IV.

Pancreatic phospholipase A_2 (EC 3.1.1.4) (reviewed in Ref. 30) is an exceptionally small enzyme (only 14 kDa) with remarkable stability with respect to heat (resists boiling for 5 min at pH 4.0), variations in pH (Fig. 3), organic solvents, urea, and guanidine [31,32].

The phospholipase A_2 activity of small intestinal aspirates stored for 3–4 weeks at room temperature was found to be completely stable. This is in contrast to the ready inactivation of the purified enzyme in the presence of trypsin at such concentrations as are present in intestinal contents [33].

A particular rich source of extracellular phospholipase A_2 is mammalian pancreatic juice, where it fulfills a digestive function by catalyzing the hydrolysis of the *sn*-2 acylester linkage of naturally and synthetic phosphoglycerides in a calcium-dependent reaction. Unlike pancreatic lipase and carboxylester lipase, this enzyme acts highly stereospecifically in that it only hydrolyzes fatty acids in a *sn*-glycerol-3-phosphate molecule (Fig. 8). The phosphate group is absolutely required, while the C1 atom of the glycerol and the fatty chain attached to it are not essential to the reaction [34].

The average western adult consumes 4–8 g of phospholipids each day. However, quantitatively more important is endogeneous phospholipid (predominantly pure lecithin) of hepatic origin (7–22 μg/day), which is secreted into the intestinal lumen via the bile [35].

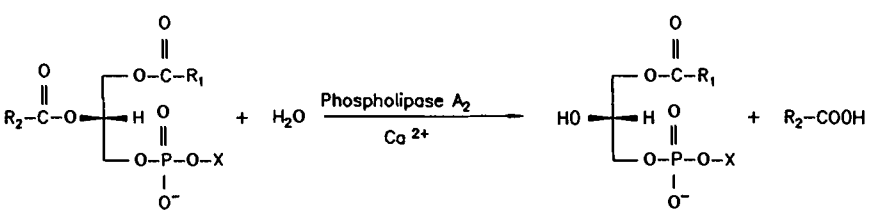


Figure 8 Specific hydrolysis of the 2-acyl ester linkage of naturally occurring or synthetic phosphoglycerides by phospholipase A_2 in a calcium-dependent reaction.

1. Molecular Properties

Like pancreatic lipase, this enzyme is clearly dependent on a lipid/water interface for maximal activity, where it also may reach a very high catalytic rate. Also like pancreatic lipase, it is not inhibited by serine esterase inhibitors like DFP or PMSF.

The pancreatic enzyme is stored and secreted as a proenzyme with an additional seven residues at the N-terminus. The proenzyme serves, like the other pancreatic proteinase zymogens, to prevent autodigestion of the pancreatic cells. Upon secretion into the gastrointestinal tract, trypsin cleaves off these seven residues to produce the enzymatic form with full activity on insoluble substrates. On monomeric substrates, however, there is little difference between the catalytic activity of the proenzyme and of the activated enzyme [34].

No precursor forms are known for the intracellular phospholipase A_2. Exploration of the three dimensional structure by means of x-ray crystallography reveals that the polypeptide chain is interconnected by seven disulfide bridges and that the majority of the amino acids are incorporated in α-helices. These features undoubtedly contribute to the high stability of the molecule [36].

2. Assay

Phospholipase A_2 activity is routinely measured by titrimetry in a pH-stat using substrate emulsions of whole egg yolk [31], purified long-chain lecithin in the presence of detergents to facilitate formation of micelles [32], and radiolabeled substrates [37].

C. Pancreatic Carboxylester Lipase

Pancreatic carboxylester lipase, secreted by the pancreas as an active enzyme without proteolytic activation, displays broad substrate specificity and has therefore received many names in the literature: carboxylesterase, bile salt–stimulated (or activated or dependent) lipase (due to its absolute requirement for bile salts to hydrolyze insoluble substrates), carboxylester lipase or hydrolase, cholesterol esterase, lysophospholipase, nonspecific lipase, and monoglyceride lipase. The IUPAC classification of the enzyme has been either EC.3.1.1.1 (carboxylester hydrolase) or EC.3.1.1.13 (cholesterolester hydrolase) (Table 2).

Recent studies indicate [38,39] that human pancreas carboxylester lipase and bile salt–stimulated human milk lipase share the same peptide sequence, also displaying a high degree of similarity to acetylcholine esterase.

1. Substrates

Typical pancreatic lipase substrates like tributyrin and triolein emulsions are hydrolyzed by carboxylester lipase in the presence of bile salt but slowly—at a rate lower than 3% and 0.5%, respectively, of that observed with the lipase-colipase complex. On the other hand, the positional specificity is not restricted: all three *sn* positions of triglycerides can be split by the carboxylester lipase. While long-chain phospholipids are resistant, short-chain phospholipids are readily attacked by carboxylester lipase [40]. The low substrate specificity of carboxylester lipase makes possible an essential role for this enzyme in the hydrolysis of triglycerides containing certain esterified polyunsaturated fatty acids, such as eicosapentaenoic, arachidonic, or linoleic acids [41], and which may be resistant to attack by pancreas lipase (see p. 190).

The various esters of cholesterol, retinoyl esters, esters of vitamin D and E, probably depend solely on the activity of carboxylester lipase for their hydrolysis. They are, before their uptake by the enterocytes, transformed into the corresponding alcohols to form mixed micelles with bile salts. Carboxylester lipase catalyzes not only the cleavage of esters but also their formation. The most studied example is the reversible esterification of cholesterol, which is favored by low bile salt concentrations and pH [42]. Additionally, the large specificity of carboxylester lipase probably functions as a first-line detoxification mechanism for a broad variety of orally ingested xenobiotics.

2. Molecular Properties

Carboxylester lipase is present in higher amounts, expressed as protein mass, than other lipolytic enzymes in duodenal juice [43]. The enzyme is coded on a polymorphic locus on human chromosome 9 [44,45] and consists of a single chain with a molecular weight varying according to the species: for example, 100 kDa for the human enzyme [46], 80 kDa for [47] the pig, and 65 kDa for the rat carboxylester lipase [48].

Although the different degrees of glycosylation contribute to the confusing enzymological positioning, the presence of a series of proline-rich repeat units also accounts for the observed interspecies variations of carboxylester lipase [49]. In contrast to pancreas lipase and phospholipase A_2, the enzyme is strongly inhibited by DFP or PMSF in the presence of bile salts [47,48]. Carboxylester lipase shares with gastric and pancreatic lipase the Gly-X-Ser-X-Gly motif at the putative active site and the Ser-His-carboxylic acid charge relay triad as catalytic mechanism. A striking homology in amino acid sequence positions demonstrates that this enzyme is close to the cholinesterases of the nervous system [49,50].

3. Effects of Bile Salts and Assay

Soluble substrates are available which are cleaved by carboxylester lipase in the absence of bile salts, such as the not quite specific *para*-nitrophenylacetate [40] or the dilaurate ester of fluorescein [51,52] (Fig. 9). However, bile salts not only protect the enzyme, but interact specifically with the enzyme, affecting approximately 7- to 10-fold the activity on water-soluble substrates like *para*-nitrophenylacetate. When water-insoluble esters like triolein or cholesterol oleate are selected as the substrate, the enzyme is virtually inactive without bile salts, which gives them the character of essential activators [53]. In contrast to pancreatic lipase, carboxylester lipase needs no cofactor like colipase and is not activated by an oil/water interface. Purified carboxylester lipase loses the majority of its activity by lyophylization but can be stored frozen at –20°C [46].

III. COMPLEMENTARY ACTIONS IN LIPID DIGESTION

Dietary lipids such as milk, fat, and egg yolk are mostly presented to the gastrointestinal tract as a triglyceride emulsion, where the globules are enveloped by phospholipids, lecithin, cholesterol, proteins, and amphiphylic peptide fragments. These surface components will determine the effectiveness of the different lipolytic enzymes involved in food digestion. For instance, colipase-lipase has on its own virtually no activity against the native milk triglyceride emulsion. Likewise, synthetic triglyceride emulsions such as Intralipid (soybean oil emulsified with egg lecithin) escape hydrolysis by pancreas colipase-lipase and bile salts.

One complementary action in physiological lipid digestion is the preincubation of the dietary fat in the stomach with the gastric lipase (Table 2). The degradation of native milk fat globules and artificial triglyceride emulsions can be initiated by this enzyme. The long-chain fatty acids generated in this first phase of lipid digestion then activate the pancreatic carboxylester lipase [54] and colipase-lipase system [55] in the duodenum, while the released short- and medium-chain fatty acids are absorbed through the gastric mucosa and become available as a source of energy to the organism. These fatty acids produced in the stomach also contribute via their antibacterial and antiviral properties to the prevention of gastrointestinal infections [56,57].

It has been demonstrated [58] that the trigger to activate the pancreatic colipase-lipase system is caused by the long-chain fatty acids released by the gastric lipase. Initial addition of long-chain fatty acids produces the same effect.

Dietary fat reaching the duodenum contains about 10–20% free fatty acids, which become partly ionized in the duodenum, where they promote fine emulsification of fat droplets and the orientation and initiation of the colipase-lipase

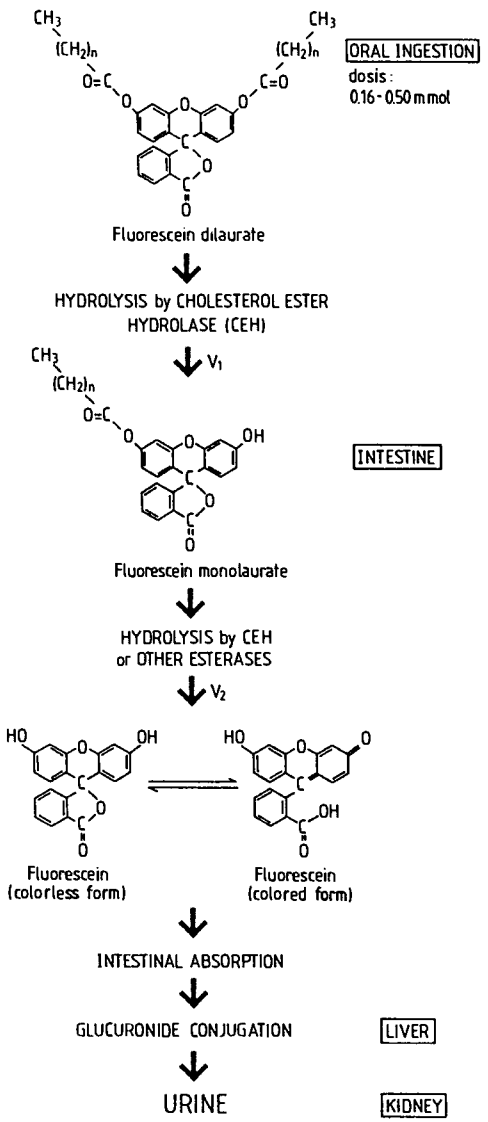


Figure 9 The pancreolauryl test allows an indirect assessing of exocrine pancreatic function. Orally administered fluorescein dilaurate is hydrolyzed by carboxylester lipase (identical to cholesterol esterase) liberating lauric acid and free, water-soluble fluorescein. The latter is readily absorbed in the small intestine, partly conjugated in the liver, and excreted in urine mainly as fluorescein glucuronide. By measuring the concentration of fluorescein in the urine over a period of, for instance, 10 hours, the total quantity of this dye is determined. (From Ref. 52.)

attack at the water/oil interface [59]. One lipolytic enzyme affecting the activity of another lipolytic enzyme occurs several times in the process of lipid digestion. The mixed lipid-bile salt micelles formed during the colipase-lipase interaction create the appropriate environment for carboxylester lipase to hydrolyze the cholesterol and vitamin esters [60]. Furthermore, the induction of carboxylester lipase activity by either phospholipase A_2 (Fig. 10) or gastric lipase [54,61] are further examples of the interaction of lipolytic enzymes. The importance of these complementary actions is further illustrated by the steep increase in pancreatic lipase activity initiated by a limited hydrolysis of the phosphoslipids stabilizing the triglyceride emulsion [35,59] (Fig. 11).

It is clear that the interconnection between the activities of the different lipolytic enzymes, where the first enzyme modifies the physicochemical state of the lipid substrate in such a way that it becomes available to another enzyme, is not only of prime importance for lipid digestion, but also results in a broad synergism between gastric lipase, colipase, pancreatic lipase, phospholipase A_2, calcium, carboxylester lipase, bile salts, and substrate intermediates [55,62–64].

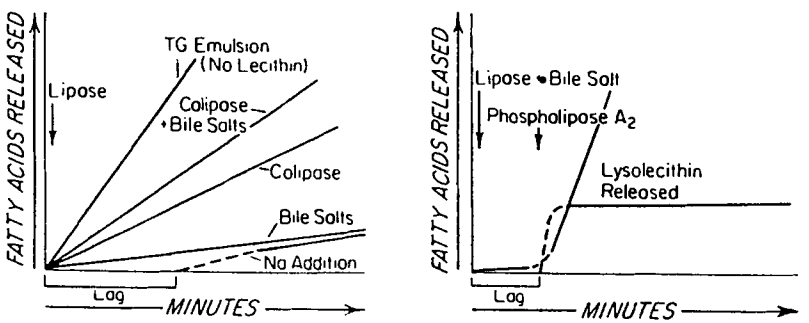


Figure 10 (Left) The rates of hydrolysis of emulsified long-chain triglycerides by pancreatic lipase. The highest rate occurs with triglycerides emulsified with gum arabic (no lecithin). All the other curves represent hydrolysis rates of triglycerides emulsified with long-chain lecithin. There is a long lag period and then a slight hydrolysis in the absence of additives. With added bile salts, the lag period is abolished, but the hydrolysis rates are still low. Both colipase and colipase plus bile salts accelerate the hydrolytic rates. (Right) Concerted action of pancreatic lipase and phospholipase. Addition of phospholipase A_2 hydrolyzes the phospholipid emulsifier (as indicated by the lysolecithin release), stops the lag phase, and initiates an accelerated triglyceride hydrolysis. (From Ref. 35.)

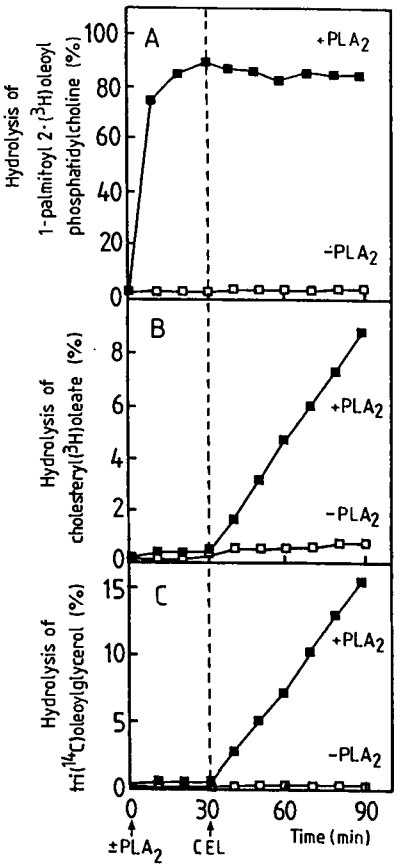


Figure 11 Hydrolysis of an artificial triolein/phospholipid/cholesterol oleate emulsion with either pancreatic phospholipase A_2 (PLA_2) and/or pancreatic carboxylester lipase (CEL) in the presence of 10 mM bile salt. Closed squares: PLA_2 + CEL; open squares: CEL alone. Hydrolysis is monitored using tritiated tracers. (From Ref. 61.)

IV. MALABSORPTION IN EXOCRINE PANCREAS INSUFFICIENCY

Patients most often requiring provision of digestive enzymes include those with chronic pancreatitis, mostly secondary to alcoholism, children and adults with cystic fibrosis, and patients with pancreatic carcinomas (Fig. 12). In addition to these conditions, pancreatic enzymes are sometimes used because of their analgesic effect for chronic pancreatitis [65].

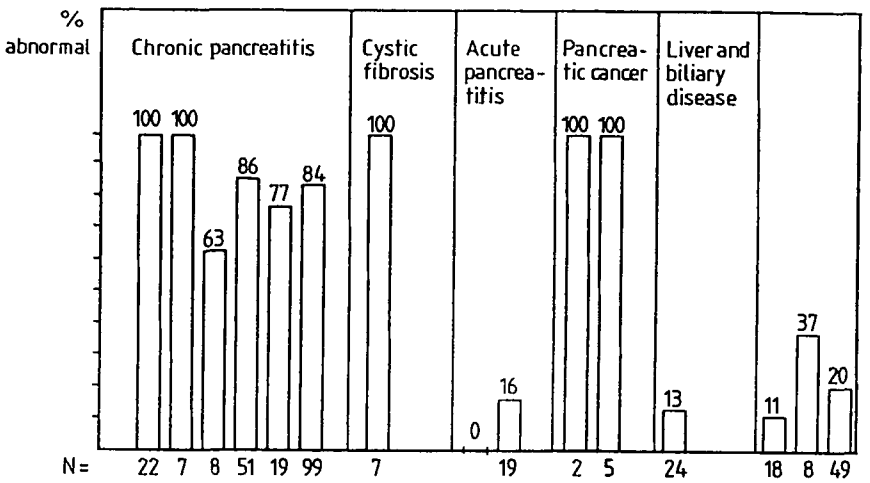


Figure 12 Degree of exocrine pancreas insufficiency in various clinical conditions evaluated by the indirect pancreolauryl test. N is the number of patients studied. (From Ref. 52.)

The final aim of exocrine pancreas substitution therapy is to eliminate malabsorption and to maintain adequate nutrition. Treatment of exocrine insufficiency of the pancreas ought to be easily achieved by administering an extract of mammalian (e.g., porcine) pancreas. Unfortunately, successful treatment is sometimes not easy to achieve and elimination of malabsorption can be difficult to maintain, notwithstanding the availability of potent porcine pancreatic extracts. Problems inherent in these pancreatin preparations themselves, like particularities in the environment in which the enzymes are expected to work, must also be considered.

The intragastric predigestion by gastric lipase [66] is not impaired in patients with exocrine pancreas insufficiency. For the completion of digestion in the duodenum, the acid gastric content must, by means of pancreatic bicarbonate, be brought to a considerably higher pH value, because the pH optimum of most pancreatic enzymes is situated in the range between 6 and 9 (Fig. 3).

This increase in pH also changes the physical behavior of the fatty acids: they become partly ionized (pK_a for long-chain fatty acids is approximately 6.4–6.6), migrate to the interface of the emulsified lipid particles, and mediate—together with bile salts, phospholipids, and cholesterol—lipid solubilization. However, in exocrine pancreatic insufficiency, a shortage of pancreatic bicarbonate secretion impairs this neutralization of pH. As a result, the postprandial

duodenal pH may remain less than 5 in chronic pancreatitis (Fig. 13) as well as in cystic fibrosis (Fig. 14).

This low duodenal pH can reduce the performance of the substitution therapy with pancreatin in several ways:

1. Enzyme inactivation: several pancreatic enzymes are irreversibly inactivated at pH 4 (Figs. 5 and 15).
2. Efficiency of lipolysis may be compromised by bile salt precipitation.
3. The substituting enzymes are too far from their respective pH optima for sufficient catalytic activity (Fig. 3).
4. Enteric-coated pancreatin preparations dissolve too late or not at all in the small intestine while the absorption of nutrients takes places chiefly in the first section of the duodenum-jejunum.

The duodenal pH also affects bile acids because of their pK_a values: 5.0–6.5 (free bile salts), 3.5–5.2 (glycine conjugates), and approximately 1.8 (taurine

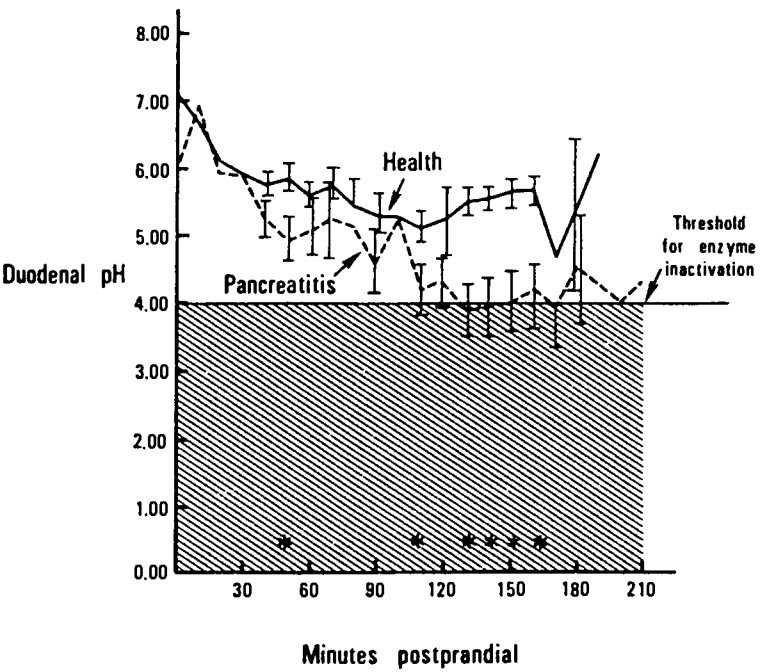


Figure 13 Postprandial duodenal pH in 6 healthy controls (solid line) and 6 patients with chronic pancreatic insufficiency (broken line). Bars show mean ± SEM; *, $p < 0.05$. (From Ref. 67.)

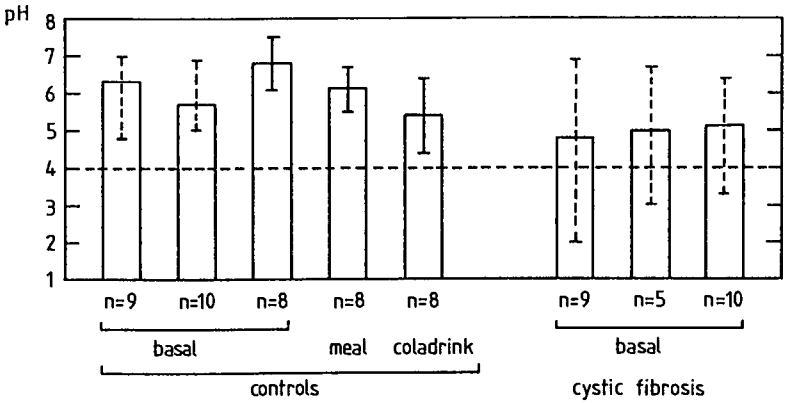


Figure 14 Duodenal pH values in control subjects during basal, meal, and drink periods (left); basal duodenal pH values in patients with cystic fibrosis (right). Irreversible inactivation of pancreas lipase occurs at pH 4. Ranges are indicated by a broken line, standard deviation by a solid line; n is the number of subjects studied. (From Refs. 68–72.)

conjugates). Undigested food components and probably a defective mucosal uptake lead to increased bile acid loss in stool and a reduced circulating pool. In response to these significant losses of bile acids, the newly synthesized acids are conjugated predominantly with glycine, which is readily available, rather than with taurine, which early becomes short in supply. Glycine-conjugated bile acids tend to precipitate easily in an acidic environment and are not further available for normal fat solubilization. Whether taurine supplementation restores the micellar solubilization of fat is not clear [73,74].

V. PANCREATIN

Pancreatin is described in the different pharmacopeia as a slightly brown, amorphous powder with a faint, characteristic odor. It is slowly and incompletely soluble in water; it is not recommended to filter the solution as the lipolytic enzymes and part of the proteolytic activity may be retained on the filter. Most pharmacopeia stipulate the minimum units of amylase (12,000 FIP units/g), lipase (15,000 FIP units/g), and protease (1000 FIP units/g) activity, but give no specifications on the content of the other components such as phospholipase A_2, carboxylester lipase, colipase, and individual proteolytic enzymes. It

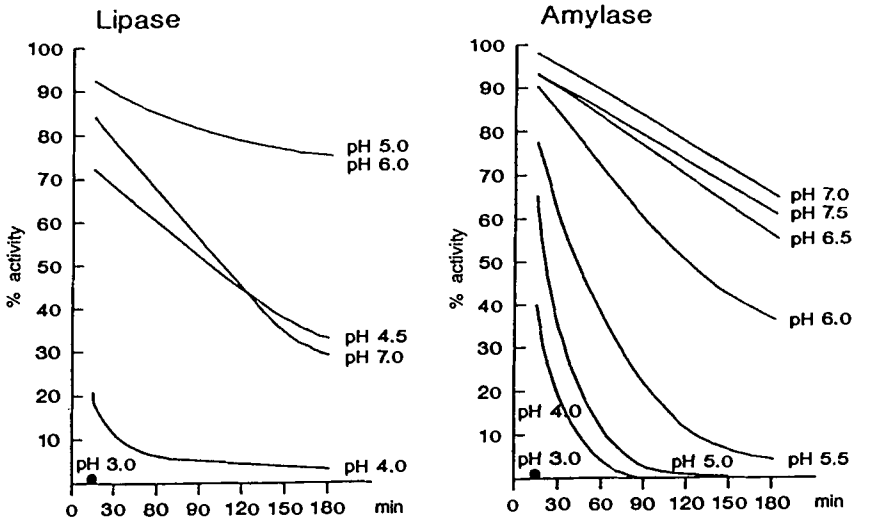


Figure 15 Stability of pancreatin lipase and α-amylase in function of different pH values and time at 37°C. Activities are measured according to the FIP procedures. (From Ref. 1.)

can be assumed that phospholipase A_2 and colipase are not destroyed during the production process. In a series of commercial pancreatin preparations a colipase-to-lipase ratio between 1.4 and 1.9 was measured [28].

As a guide [1], the following approximate equivalencies between the FIP, British Pharmacopoeia, European Pharmacopoeia, and U.S. Pharmacopeia units can be assumed:

For lipase: 1 FIP unit ≈ 1 Europ P unit ≈ 1 BP unit ≈ 1 USP unit
For amylase: 1 FIP unit ≈ 1 Europ P unit ≈ 1 BP unit ≈ 4.15 USP unit
For protease: 1 FIP unit ≈ 1 Europ P unit ≈ 1 BP unit ≈ 62.5 USP unit

The final quality of a pancreatic preparation produced depends not only on the strain and feeding of the pigs from which the pancreatic glands are collected, but also on the different steps to remove ballast substances, fat, water, and microbial contamination [1].

Using optimized procedures, up to about 11,000 FIP units of lipase can be extracted from 1 g of pancreas gland. Some pancreatin preparations may contain relatively important amounts of purines, which in patients receiving large doses of drugs can lead to hyperuricosuria and hyperuricemia [75].

A. Unprotected Pancreatin Preparations

When pancreatin is administered to patients in some galenic formulation, it mixes with the food mass and performs some digestion in the stomach. However, most enzymic activities will be destroyed before reaching the duodenum [76]. Only therapeutic results can be expected in patients where the postprandial pH remains relatively high as a consequence of, for example, alcoholic gastritis or concomitant medication with inhibitors of gastric acid secretion.

B. Enteric-Coated Pancreatin Preparations

Enteric coating may be a favored approach to circumvent gastric inactivation of pancreatic enzymes. A coating that is impervious to acid but dissolves at a more neutral pH is used to protect the enzymes during their passage through the stomach. Enteric-coated preparations should not disintegrate and release their content until a defined pH value, e.g., 5.6 [77] (Fig. 16), is encountered. To be effective, the gastric contents may not be brought, even temporarily, above this value by the food mass. On the other hand, dissolution of the coating in the duodenum will require a pH of 5.6 or more in that medium. Therefore, it is possible that in patients with exocrine pancreas insufficiency, due to the low output of bicarbonate, the period of time during which the pH of the duodenal content reaches a value of more than 5–6 will be too short to allow adequate dissolution of the coating. It is therefore possible that coated pancreatin preparations will be released too late and at a too distal location in the intestinal tract to be effective. Besides having the proper dissolution characteristics, the galenic formulation should not only thoroughly mix with the contents of the stomach, but also move quickly and simultaneously with the predigested food mass to the duodenum. A diameter ranging, respectively, between 1.1 and 1.7 mm for microspheres with a density of 1 [78] and 1.0–1.2 mm [79] has been proposed for this purpose.

To about 10% of the normal pancreatic secretion, 100,000 FIP units of lipase are required per meal [80]. This means that in order to facilitate patient compliance, the galenic formulation should contain the maximum amount of pancreatin possible per dose. Earlier studies suggest a minimum of 30,000 FIP units per meal [81], but in recent years higher amounts per meal have been recommended [80,82].

VI. ADJUNCT THERAPY WITH INHIBITORS OF GASTRIC ACID SECRETION AND ANTACIDS

Several pharmacological attempts have been made to optimize the intraduodenal pH by diminishing free acid in the stomach. In addition to histamine H_2 receptor

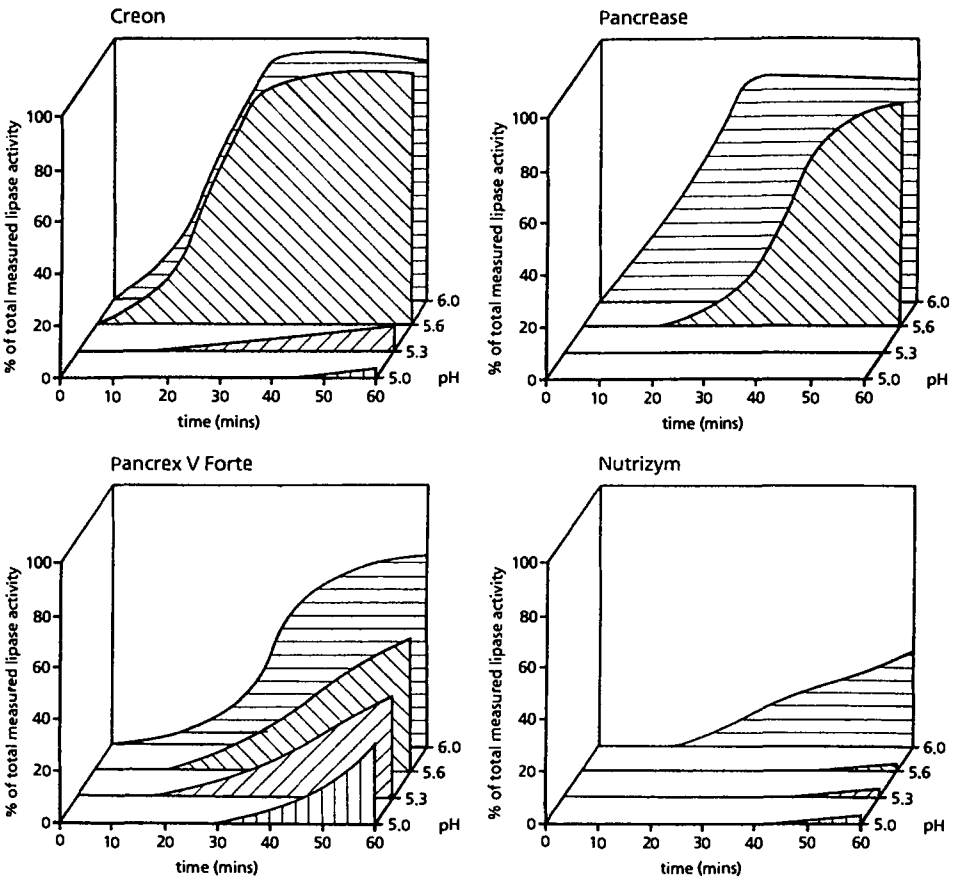


Figure 16 pH dissolution characteristics of some commercial enteric-coated pancreatin preparations expressed as rate of release of lipase activity between pH 5 and 6. (From Ref. 77.)

blockers, metiamide [83], cimetidine [84–86], and ranitidine [87], as well as the H^+/K^+-ATPase inhibitor omeprazol [88,89] and the prostaglandin E_1 analog misoprostol [90], have been included in clinical studies in combination with pancreatin preparations. Results seem to be encouraging, but serious side effects have been observed, and a balance between the positive effects on increased intraduodenal enzyme activities and the physiological demand of acidity in the stomach must be sought.

The side effects inherent to some of these drugs used as lifelong treatment must be taken into account. Also, reduced gastric acidity eliminates a natural physical protective barrier and may cause, with continuous use, bacterial overgrowth [91] and gastroenteral infections [92]. This would be especially problematic for cystic fibrosis patients, who, because of the nature of their disease, are more susceptible to intestinal infections.

A combination of pancreatin with antacids such as bicarbonate seems not to result in improved effects [93–95]. This may be attributable to reflex stimulation of acid secretion, binding of bile acid by calcium, or formation of calcium and magnesium soaps with the liberated fatty acids. Furthermore, high doses of bicarbonate are considered unpalatable, especially by children.

The use of gastric lipase (Table 2), if it can be done at a reasonable cost, should also be considered. However, its isomeric specificity, restricted mainly to the *sn*-3 position, makes such an approach unlikely.

VII. BENEFIT ANALYSIS

The heterogeneity of the clinical picture in cystic fibrosis is reflected in the large number of mutations elucidated in the large-size gene encoding for the cystic fibrosis transmembrane conductance regulator (CFTR). For the severity of pancreatic insufficiency in cystic fibrosis, an overall genotype-phenotype correlation has been demonstrated, while for the respiratory symptoms, probably due to environmental and other genetic factors, this genotype-phenotype association is less evident.

Enzyme replacement for exocrine pancreas insufficiency must be adjusted on an individual basis considering age, body weight, weight for height, growth rate, fat intake, supplementary energy needs (particularly for pulmonary function), and clinical as well as laboratory evidence of malabsorption. Clinical symptoms include abdominal pain, distension, intolerance of fatty foods, and character of stools (consistency, number, odor). A more objective assessment involves microscopic examination of stool for neutral and split fats, measurement of fecal chymotrypsin or elastase [96].

A periodic measurement of stool fat excretion by means of the method of Van de Kamer [97] or using the less unpleasant near-infrared analysis [98–100] allows quantitative evaluation of the degree of steatorrhea. Besides this, several function tests where the pancreas is directly stimulated by an infusion of secretin and/or cholecystokinin (reviewed in Ref. 101) or indirect tests requiring the administration of an exogenous pancreas enzyme substrate as *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid (NBT-PABA) or fluorescein dilaurate (pancreolauryl test) (Fig. 12) are available. The NBT-PABA test measures intraluminal

chymotrypsin activity, whereas the pancreolauryl test evaluates carboxylester lipase activity. In specialized centers, a triglyceride breath test using an isotopic labeled fatty acid or an unusual fatty acid can also be considered [101–103].

The dose of pancreatic supplement is usually gradually increased during the initial stages of treatment until objective measures of fat absorption indicate a 90% fat uptake. In practice, the dose of pancreatin for each meal is divided into two parts, with half given at the start of the meal and half at the middle. Enzymes must also be taken with snacks, including milk. Children can often swallow capsules by the age of 5 years. For younger children, the enzymes are taken out of the capsule and mixed with liquid, fruit puree, or soft fruit.

In patients whose maldigestion is difficult to control, other underlying conditions should be considered, such as noncompliance, infections of the gastrointestinal tract, celiac disease, lactose deficiency, inflammatory bowel disease, anatomical abnormalities, diabetes, and liver cirrhosis. This question is complicated by the abundance of small intestinal viscous glycoproteins in cystic fibrosis, prolonged intestinal transit times, which may provoke bacterial overgrowth [104], and absorption of the enzymes in their macromolecular form [105].

VIII. CURRENT ISSUES AND PROSPECTS

A general principle of treatment is to maintain patients with the lowest dose of enzyme replacement that provides the desired therapeutic effect. However, in patients who need large doses of pancreatic enzymes, a reduction of the number of doses necessary improves compliance and daily comfort. On the other hand, the possible association between colonic strictures (fibrosing colonopathy) in children with cystic fibrosis and intake of high-strength microsphere preparations of pancreatic enzymes is clearly dose related and possibly brand related [106]. The use of laxatives or prokinetic agents, such as cisapride, which will deliver intact enzymes to the colon more rapidly, appears to be an additive factor. Which of the constituent enzymes, or perhaps their enteric coating, is the primary noxious agent is still controversial. Considerable variation can occur in different commercial pancreatic enzyme supplements, such as (1) enzyme content per dose unit, (2) ratios of pancreatic lipase to α-amylase and proteases, (3) particle size of microspheres, (4) manufacturing process of the enteric coating, and (5) excess of enzyme over the stated activity in order to guarantee a minimum level of activity at the end of shelf life.

The regulatory status of pancreatin preparations is different in the United States than in European countries. Because pancreatic supplements were available before the U.S. Food, Drug and Cosmetic Act of 1938, they are at this moment not under obligate FDA approval. However, a series of reports [107] on

fibrosing colonopathy occurring with recently introduced high-strength enteric-coated pancreatine supplements may provoke a discussion as to whether new preparations should be given Investigational New Drug status.

Relevant quality control should not be restricted to the usual triad of activities of pancreas lipase, α-amylase, and trypsin, but should be extended to the content of colipase, the activities of the two other lipolytic enzymes present in pancreatine (phospholipase A_2 and carboxylester lipase), and the dissolution characteristics of enteric-coated preparations as a function of time and pH (Fig. 16). The availability of such information will certainly contribute to a better tailoring of the management of maldigestion in the individual patient and to a more appropriate correction of the obligate nonphysiological route of delivery of these enzyme supplements.

Genes of different human digestive enzymes as well as those of several acid-stable microbial enzymes have been cloned and expressed. Some survive gastric proteolytic and acid denaturation and maintain their enzymatic activity in the complex physiological microenvironment of the lumen of the small intestine, being mainly conditioned by pH and bile acids [108–110]. They may be useful in the therapy to pancreatic insufficiency, but require confirmation of clinical efficacy in carefully designed protocols in different age groups.

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l-Asparaginase: An Essential Antineoplastic Drug

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I. DISCOVERY OF l-ASPARAGINASE AS AN ANTINEOPLASTIC DRUG

The earliest observation that turned out to be important for the development of l-asparaginase (l-ASP; l-asparagine aminohydrolase, E.C. 3.5.1.1) as a therapeutic agent against malignant tumors was made in 1922 [1]: Clementi showed that guinea pig serum contained a high activity of l-ASP. This phenomenon has exclusively been observed in guinea pigs, while other mammals are almost devoid of this enzyme.

In 1953 Kidd [2] attempted to inhibit growth of a cell line of Gardner's lymphoma, so-called 6C3HED cells, using a specific rabbit antibody supplemented with guinea pig serum as a source of complement. Tumor growth in vitro was inhibited, but the same effect was reproduced using guinea pig serum alone in the absence of rabbit antibody. Sera derived from other species of animals (i.e., dog, rabbit, rat) were unable to induce tumor effects. The author concluded that a serum component exclusively present in guinea pig serum was responsible for tumoricidal effects [3]. These observations were extended in 1956. Neuman and McCoy [4] demonstrated that growth of a cell line derived from the Walker carcinosarcoma was dependent on l-asparagine. Similar work was published by Haley and coworkers using a mouse leukemia cell line [5]. It was further shown that growth of normal cells was not dependent on l-asparagine, making l-ASP a potential tumor-specific drug. Other cell lines which are not consistently dependent on l-asparagine are reviewed by Gallagher et al. [6].

The first scientist to show that l-ASP purified from guinea pig serum was in fact a tumoricidal agent against implanted 6C3HED cells was Broome [7]. Serum from newborn guinea pigs lacking l-ASP was devoid of antitumor activity. Further studies by the same author confirmed its therapeutic potential [8,9]. Using salt precipitation, starch block electrophoresis, and DEAE cellulose chromatography, two isoforms of l-ASP were partially purified. Purification to homogeneity was achieved by Yellin and Wriston [10]. Interestingly, only one isoform had antilymphoma activity in vivo [11].

Unfortunately l-ASP was difficult to obtain from guinea pig serum in sufficient amounts. Thus, other potential sources for the enzyme had to be found. Mashburn and Wriston [12] successfully purified *E. coli* l-ASP and demonstrated its tumor-inhibitory activity, which opened the way for large-scale production of the enzyme.

The next step from a cell line to animal experiments was done by Old and coworkers [13]. Antitumor activity was demonstrated in vivo in a dog model with spontaneous lymphosarcoma. In the same year Oettgen and collaborators were the first to show similar efficacy of l-ASP in humans with leukemias [14]. Since then large numbers of clinical trials have been performed using the enzyme. It turned out that l-ASP is not an anticancer drug in general but that it is effective against certain types of tumors. For these it has become an essential component of chemotherapy strategies.

II. TYPES AND CHEMICAL STRUCTURE OF l-ASPARAGINASE AVAILABLE FOR THERAPY

A. Unmodified Enzymes

The first efficacy studies were performed with guinea pig l-ASP, but bulk preparation of the enzyme turned out not to be feasible. Large-scale production could only be achieved using bacteria (*Escherichia coli, Serratia marcescens, Erwinia carotovora = Erwinia chrysanthemi, Pseudomonas stutzeri*). Presently two major sources are still in use: *E. coli* and *E. chrysanthemi.* Further attempts have been made to reduce the immunogenicity of the drug by covalently linking the enzyme with polyethylene glycol (PEG). As this modified drug seems to have a unique therapeutic potential, it will be discussed separately.

Using different strains of *E. coli,* different isoenzymes of l-ASP have been isolated [15]. The molecular weight was found to be 120 kDa with some isoenzymes having 240 kDa but lower specific enzyme activity. All asparaginases are obviously composed of four subunits whose molecular weight was initially

reported to be 22 kDa [16]. Our own observations are in slight contrast to these reports [17]. Elution profiles obtained with four different preparations using HPLC and a TSK SW 4000 blue column are shown in Figure 1. Three preparations have similar characteristics, while PEG-l-ASP has a molecular weight markedly higher than the others (220 kDa). Figure 2 shows results of SDS-PAGE applied to the three non-PEGylated enzymes. Major bands most likely representing the major enzyme subunits are found around 32 kDa for the *E. coli* preparations and around 40 kDa for the *Erwinia* preparation. Using the same conditions the behavior of the PEGylated enzyme is completely different (Fig. 3). The major band has a molecular weight > 200 kDa, which we believe is due to cross-linking of the subunits by polyethylene glycol. A summary of our results on protein chemistry is given in Table 1.

Specific enzyme activities are markedly different among preparations; Table 2 gives a brief summary [18]. It seems important to mention l-ASP also contains up to 10% l-glutaminase activity (Table 2). As all purification procedures attempting to get rid of this activity have been unsuccessful so far and even recombinant l-ASP has l-glutaminase activity, it seems likely that hydrolysis of l-glutamine is not mediated by a contaminating enzyme but by l-ASP itself [19].

The crystal structure of *E. coli* l-ASP II was determined [20] at 2.3 Å resolution. l-ASP was found to be a homotetramer with 222 symmetry belonging to the class of α/β proteins. Each subunit expresses two domains with the active site located in between.

Genes encoding *E. coli* l-ASP have been cloned for both the secreted high-affinity enzyme l-ASP II and the low-affinity cytoplasmic enzyme l-ASP I. The latter is constitutively produced, while l-ASP II expression is positively regulated by both a cyclic AMP receptor protein and anaerobiosis. Deduced amino acid sequences of l-ASP I shows discernible similarity to l-ASP II in a region immediately adjacent to the proposed active-site peptide of l-ASP [21]. l-ASP I is encoded by the gene ansA and l-ASP II by ansB, the latter gene having been cloned by Jennings [22]. The deduced amino acid sequence of l-ASP II contained differences at 11 positions when compared with a previously determined amino acid sequence [23]. The predicted molecular weight was 4×35.6 kDa. Similar results were obtained by Bonthron [24] using a cloning strategy based on the polymerase chain reaction.

For industrial purposes expression systems that overproduce l-ASP II from recombinant plasmids must be developed. Appropriate expression systems produce l-ASP II at 10–15% of total cellular protein [25]. Purification of the enzyme is achieved by a two-step procedure. The recombinant product seems to be identical to conventionally purified preparations as far as biochemical properties are concerned. Antitumor activity remains to be established.

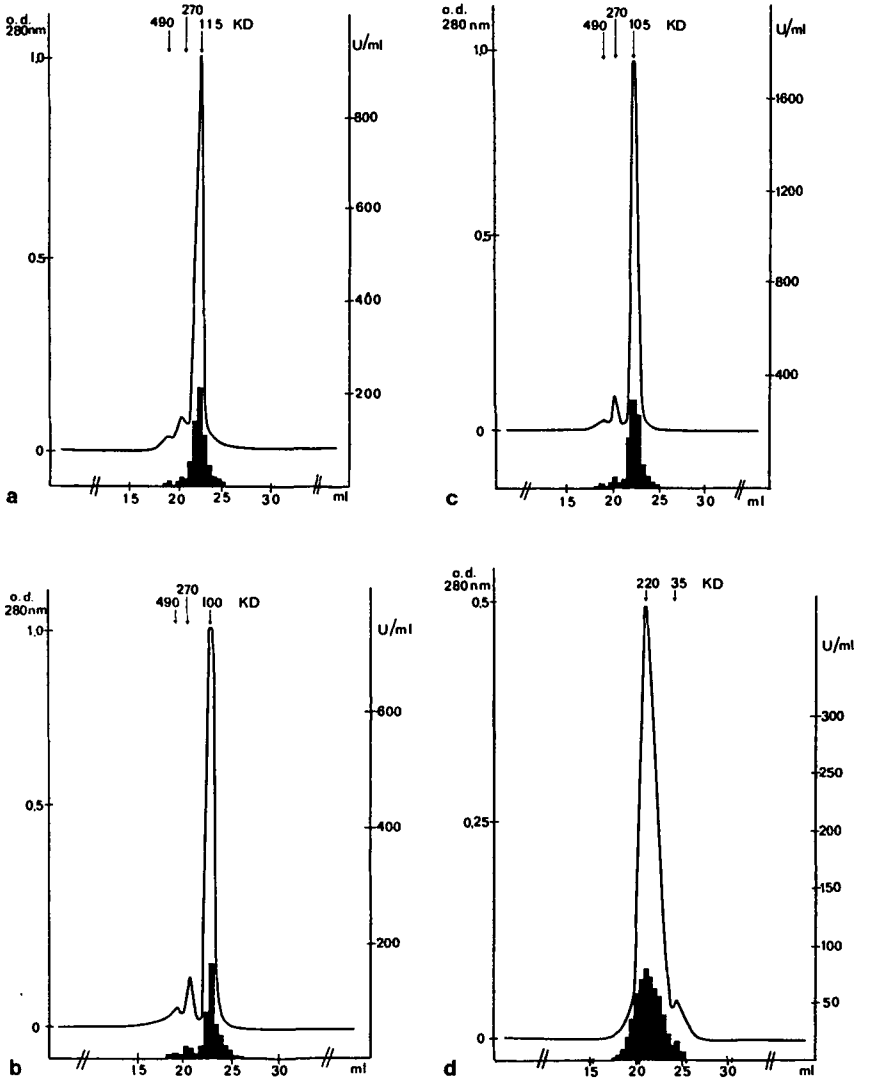

Figure 1 Elution profile of 2.5 mg l-ASP preparations on a TSK SW 4000 blue column (Pharmacia-LKB, 7.5 × 60 mm). The flow rate was 60 mL/h; each fraction contained 0.5 mL. Protein concentration is recorded automatically. The enzyme activity (U/mL) was determined in each individual fraction. Molecular weight markers are indicated by arrows. (a) Crasnitin from Bayer, Leverkusen, FRG; (b) Leunase from Kyowa, Hakko, Kogyo Co. Ltd., Tokyo, Japan; (c) Erwinia-Asp from The Centre for Applied Microbiology, Salisbury, U.K.; (d) PEG-modified *E. coli* l-ASP from ENZON, South Plainfield, NJ.

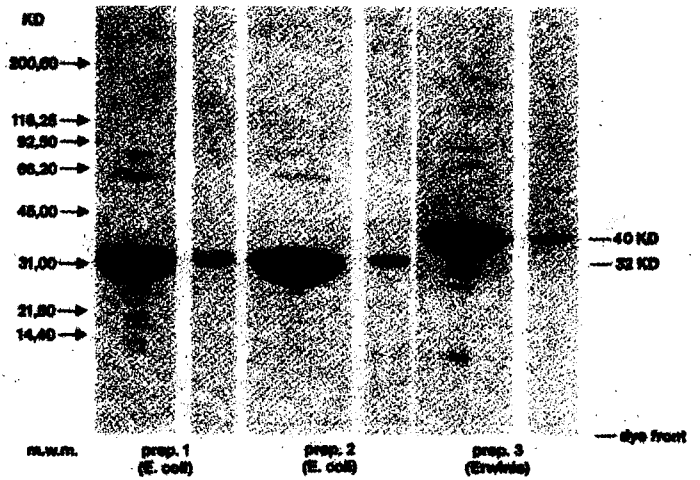

Figure 2 SDS-PAGE or preparation I and II (100 and 2000 U/mL) as well as preparation III (250 and 5000 U/mL) using 5–20% gel and nonreducing conditions. Samples were boiled in sample buffer. Molecular weight markers are indicated by arrows on the left.

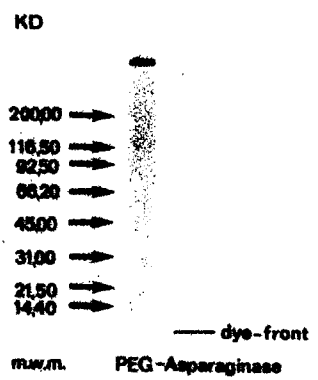

Figure 3 SDS-PAGE if PEG-modified l-Asp (500 and 100 U/mL) using a 2–20% gel and nonreducing conditions. Molecular weight markers are indicated by arrows on the left.

Table 1 Protein Composition of Four Different l-Asp Preparations Separated by Gel Chromatography

Preparation	Molecular weight (Kd)	Enzyme activity (U/mg)	Relative protein content (%)
E. coli I	490	111	5.1
	270	148	8.4
	115	133	86.5
E. coli II	490	131	5.2
	270	150	10.3
	100	128	84.5
Erwinia-Asp	490	251	2.2
	270	308	5.3
	105	265	91.5
PEG-Asp	220	120	87.5*
	35	153	12.5*

*Little variability in different lots of PEG-Asp.

Another gene encoding *Saccharomyces cerevisiae* has recently been characterized [26]. It may well be that in the future, using recombinant gene technology, more alternatives to *E. coli* or *Erwinia* l-ASP will become available.

B. Chemically Modified Enzymes

Parenteral therapy with foreign proteins in humans has limitations due to the fundamental problem of the drug's immunogenicity. As real immunological

Table 2 Molecular Weights, Specific Enzyme Activity, and Glutaminase Activities of Four Different l-ASP Preparations

l-ASP source	Molecular weight (kDa)	Specific activity (U/g)	Glutaminase activity (%)
Guinea pig	130	50	0
E. coli	130	270	2
Erwinia	130	700	10
Serratia	150	60	5

Source: Ref. 18.

tolerance that would require antigen-specific T-cell–mediated immunosuppression is difficult to achieve, other approaches have been made toward immunological nonresponsiveness rather than tolerance. One way to overcome the problem for a limited period of time is to switch to another preparation that is not cross-reacting. The other way is chemical modification of the enzyme, which reduces its immunogenicity but preserves the enzyme activity.

1. Enzymes Modified with Polyethylene Glycol

Abuchowski [27] successfully coupled PEG to l-ASP. Its antileukemic activity was shown in the L5178Y tumor-bearing BDF mouse model. Coupling of the enzyme to PEG almost abolished the drug's immunogenicity. This observation was confirmed by Japanese researchers [28]. The molecular weight of PEG used and the degree of modification of the enzyme turned out to be crucial to obtain optimum results. Biochemical properties of PEG-l-ASP markedly differ from the native enzyme. Its apparent molecular weight is higher (Fig. 1). SDS is unable to separate subunits of the enzyme (Fig. 3), probably due to cross-linking by PEG. Its reactivity with specific antibodies is very low but increases if the drug is subjected to freezing-thawing cycles [17]. Available but still limited clinical experiences indicate that the PEGylated enzyme has antitumor activity in both animals [27,28] and humans [28,30]. Its low immunogenicity has been confirmed in vivo even in highly sensitized children with multiple acute lymphoblastic leukemia (ALL) relapses [27,29].

2. Enzymes Modified with Dextran

l-ASP has been coupled to dextran in order to improve thermal stability, provide resistance to proteolytic digestion, prolong half-life, and reduce immunogenicity. Various products have been generated depending on dextran molecular weight (reviewed in Ref. 31). The molecular weight of dextran also determines the degree of enzyme inactivation (up to 65%). Reduction of immunogenicity by dextran seems to be less effective than by PEG.

3. Enzymes Modified with Polyethylene Glycol and Maleic Anhydride

Kodera et al. [32] used a comb-shaped copolymer of polyethylene glycol derivative and maleic anhydride to abolish the enzyme's immunogenicity. Two different products were obtained—one having 85% of the original enzyme activity the other 46%. Following the modification process there was no longer demonstrable immunoreactivity toward anti-l-ASP antisera. In vivo studies have not been performed.

4. Enzymes Modified with Poly-DL-Alanyl Peptides

A second procedure to reduce l-ASP immunogenicity has been described by Uren and Ragin [33]. They used poly-DL-alanyl peptides to block immunogenic epitopes within the l-ASP molecule derived from both *E. coli* and *Erwinia.* Similar to the PEGylated enzyme, the half-life in mice was markedly prolonged compared to the native enzymes even if the animals had previously been immunized with the latter preparation. Hyperimmune animals, however, rapidly cleared both the native and modified enzymes. Clinical studies have not been performed to date.

5. *Erwinia* l-ASP Modified with Human Albumin

Another approach for enzyme modification has been made by Nerkar [34] by coupling *Erwinia* l-ASP with human serum albumin. The complex was enzymatically active but had a 10-fold lower Km value. In vivo studies have not been performed.

6. Acylation

Acylation has been applied as a method for l-ASP modification [35]. Following this procedure the enzyme becomes rather hydrophobic. Neither pharmacokinetic nor immunological data are available.

7. Drug-Delivery by Erythrocytes

Another method for drug delivery is l-ASP entrapped in erythrocytes [36]. The enzyme was stable within red blood cells and the half-life in vivo markedly prolonged [36,37]. Intravenous infusion in mice resulted in an effective elimination of l-asparagine for a period of 2 weeks [38]. Studies in humans by the same author suggest the possibility of reducing intolerance reactions by this method, however, the immunogenicity of the enzyme seems to be only slightly diminished [39]. Recently evidence was provided that doses of 150–200 IU/kg body weight (bw) resulted in effective elimination of l-asparagine for 50 days [40].

8. l-ASP Delivery with Liposomes

l-ASP entrapped in liposomes can be expected to display reduced immunogenicity and toxicity. Studies with 6C3HED lymphoma cells implanted intraperitoneally [41] and subcutaneously implanted lymphoma [42], unfortunately, revealed reduced effectivity against these tumors. This problem has recently been overcome. Encapsulation of l-ASP in small liposomes with a median diameter of

158–180 nm resulted in marked prolongation of in vivo half-life in animals, avoidance of induction of l-ASP antibodies, and enhanced antitumor activity [43].

Palmitoyl-l-ASP, a chemically modified derivative of the enzyme encapsulated in liposomes, has also been studied in animals [44] and shown to exhibit prolonged half-life (about 10-fold), no acute toxicity, but preservation of in vivo antitumor activity.

9. Recombinant l-ASP

Moola et al. [45] studied epitopes on *E. chrysanthemi* using synthetic hexapeptides and polyclonal antisera from rabbits and mice. Elimination of immunodominant epitopes in the enzyme by site-directed mutagenesis resulted in markedly decreased binding of the antibodies indicating reduced immunogenicity while enzymatic activity compared to the wild-type enzyme remained unchanged. The enzyme was expressing in *E. coli.*

III. MECHANISM OF ACTION

A. Antineoplastic Action

l-ASP converts l-asparagine to l-aspartic acid. As several types of tumor cells require l-asparagine as an essential amino acid for protein synthesis, they are deprived of an essential growth factor in the presence of l-ASP. Effective depletion of l-asparagine results in cytotoxicity for leukemic cells (Fig. 4). Asselin et al. [46] have quantified cell kill both in vitro and in vivo in patients with ALL undergoing treatment with l-ASP as a single agent. Using flow cytometry and dye exclusion methods, they were able to show that l-ASP effectively kills leukemic blasts in vitro and in vivo. However, not all patient cells were equally sensitive. To date the authors have not analyzed whether or not a poor l-ASP response is associated with a higher relapse rate on follow-up.

Besides these specific effects, l-ASP can exert immunosuppressive effects in general. Using an animal model, Chakrabarty [47] demonstrated depression of both humoral and cellular immune reactions. In mice rejection of transplanted graft was found to be delayed [48,49]. Also in mice under the influence of l-ASP, a decreased immune response to neoantigens was shown [50].

PHA-induced blastogenesis in murine 6C3HED cells as well in human acute lymphoblastic leukemia cell lines is markedly inhibited by asparaginases, the effect being 10-fold higher for the *Erwinia* enzyme [51]. Similar results were obtained by Ashworth et al. [52] using PHA responses of rabbit blood leukocytes in vitro. It seems possible that the relatively higher glutaminase activity (Table 2)

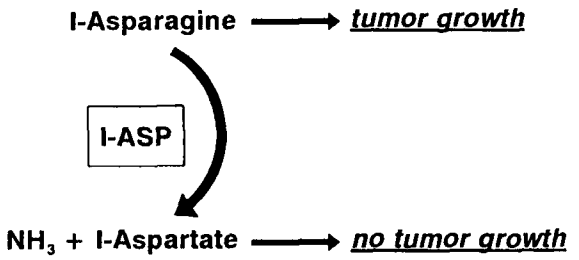

Figure 4 Mechanism of action of l-asparaginase (l-ASP). The growth of l-asparagine–dependent tumors is inhibited if this essential substrate is hydrolyzed to ammonia and l-aspartate.

is responsible for this effect because l-glutamine is an important growth factor even for normal cells in culture.

While these in vitro results indicate significant differences among preparations in vivo, immunosuppressive effects in animals are comparable. Studies in humans addressing the question of possible immunosuppressive effects have not been performed so far. It seems unlikely that such studies will ever be performed in the future because none of the current treatment protocols uses l-ASP as a single agent. It is always part of multiple agent regimens and, thus, combined with drugs with definitive immunosuppressive effects. But even if immunosuppression occurs in humans, it is not profound enough to suppress anti-l-ASP immune responses (see below).

B. Resistance

Tumor cells can develop the potential to newly synthesize l-asparagine intracellularly, which makes the cells resistant to the action of l-ASP [9]. Mechanisms that lead to derepression of the gene for asparagine synthetase have not yet been fully elucidated. Some progress toward understanding these events comes from the work of Andrulis and Barrett [53] and Greco et al. [54]. They showed that the degree of methylation of cytosine residues in DNA regulates the expression of asparagine synthetase. Drugs that inhibit cytosine methylation like 5-azacytidine increase enzyme expression, while others like ARA-C increase methylation and by this inhibit enzyme expression [55,56]. This may be the reason for the clinically observed synergy between high-dose ARA-C and l-ASP [57].

Accelerated clearance of l-ASP following induction of specific antibodies has also been found as a potential reason for resistance [58]. However, other

groups reported that despite immunization of patients, the drug's efficacy remained unimpaired [59].

Gallagher et al. [6] speculate on another mechanism: the pool of l-ASP–sensitive cells may produce cytokines that control the expansion of resistant cells. As soon as sensitive cells are killed by l-ASP, resistant cells escape from regulatory control.

IV. ANIMAL STUDIES

Some animal studies have already been mentioned (Sec. I). In this section, further details of animal experiments will be briefly summarized.

A. Pharmacokinetics of Native Enzyme

Pharmacokinetics in animals depend on the species studied as well as the route of administration. Table 3 gives a brief overview.

B. Pharmacokinetics of PEG-l-ASP

This part of the chapter will be restricted to PEG-l-ASP because this type of modified l-ASP seems to be the most promising for clinical studies. However, it should be mentioned that PEG modification is not standardized. The half-life varies depending on the molecular weight of PEG used as well as the degree of protein modification [31]. Table 4 describes the effect of PEGylation on half-lives in some animals.

Table 3 Approximal Half-Lives of l-ASP in Hours in Different Experimental Animals

Host	Route	*E. coli*	*Erwinia*
Mouse	i.p.	2.8–4.2	4
Guinea pig	i.p.	4.5	4.5
Rat	i.p.	1.5	1.8
Dog	i.p.	14–23	13
Rabbit	i.v.	9	10
Sheep	i.v.	5	2
Monkey	i.v.	8	4

Source: Modified from Ref. 30.

Table 4 Effect of PEGylation of l-ASP on In Vivo Half-Lives in Experimental Animals

Source of l-ASP	Route	Host	Half-life (h)	
			Native	PEGylated
Achromobacter	i.v.	BDF1 mice	2	24
E. coli	i.p.	Rats	2.9	56
E. coli	i.v.	Mice	3.35	32.9
E. coli	i.v.	Rabbits	20	144

Source: Modified from Ref. 30.

C. Efficacy of Native Enzyme

Unmodified l-ASP was found to have antitumor activity against more than 50 mouse neoplasms, rat Murphy-Sturm, canine lymphosarcomas, rat fibrosarcoma, Walker carcinosarcoma, and Jensen sarcoma [60–62].

D. Efficacy of PEG-l-ASP

Most recent efficacy studies in animals employed the dog lymphosarcoma model. Yoshimoto et al. [28] were able to induce remission in a dog without recognizable side effects. This first anecdotal observation could later be replaced by extended trials. Teske et al. [63] performed a randomized trial in 42 dogs with non-Hodgkin's lymphoma. The efficacy of PEG-l-ASP was equal to conventional l-ASP. Side effects, however, were absent in the PEG-l-ASP group but frequent and severe in the group receiving the conventional drug. The most frequent side effect was anaphylactic shock. In 1992 McEwen et al. [64] performed a randomized double-blind study in 69 dogs with canine lymphoma comparing the conjugate with the unmodified drug. Again, the efficacy of both drugs was comparable. Toxicity was low in both groups of dogs but tended to be higher with the conventional drug.

V. HUMAN STUDIES

A. Pharmacokinetics of Native Enzyme

Pharmacokinetics very much depend on the route of administration (i.m. or i.v.). Schwartz et al. [65] showed that the in vivo half-life is not only related to the route of administration but also to the preparation used. After i.v. injection the in

vivo half-life was 7.5 hours for one preparation (Bayer) but two- to threefold longer for the others (Merck, Squibb). In vivo half-life of *Erwinia* l-ASP is similar to the *E. coli* preparation (mean 10 h) [66].

Schwartz also addressed the question whether or not the cerebrospinal fluid (CSF) can be reached by the drug. Even after i.v. administration of 5000 U/kg bw, only small amounts of l-ASP were detectable in CSF. When the enzyme was injected directly into the CSF, it was rapidly transferred to plasma. Thus, clinical effects after i.t. injection can only be expected up to 24 hours. Pütter [67] determined in vivo half-life of *E. coli* l-ASP as 12 hours, while it was 24 hours for another preparation (Asnase X). These results were obtained after i.v. injection.

Ho and collaborators [68] injected 10,000 U/m^2 i.m. or 16,500–100,000 U/m^2 i.v. of *E. coli* l-ASP into adults with metastatic cancer or leukemia. A plateau in plasma after i.m. injection was not reached until 14–24 hours after drug administration. The peak plasma level was 1.12 U/mL, about one fourth of the activity reached when the same dose was given i.v. The in vivo half-life was 46 hours (after i.m. application) compared to 7–28 hours for the i.v.-administered drug. The volume of distribution after i.m. administration was four times that after i.v. administration. Consequently, the area under the curve after i.m. administration was only 1/24 that by i.v. route.

Our group [69] studied in vivo half-life in 24 children (2–15 years) with acute lymphocytic leukemia under multiple agent therapy including *E. coli* l-ASP at 3000 U/kg bw i.v. The $t_{1/2\beta}$ ranged from 3 to 14 hours (median approx. 6 h). Surprisingly, half-life in children having demonstrable antibodies to l-ASP prior to infusions tended to be longer than half-life in nonimmunized children. However, for this observation the level of significance was not reached. Other groups found that in vivo half-life is markedly reduced in individuals with detectable l-ASP antibodies [58,70].

As l-ASP is not detected in the urine, it is most likely eliminated by the reticuloendothelial system (RES). Some authors speculated that it might be proteolytically degraded by proteases like cathepsin [67]. If so, it is reasonable to assume that the molecular weight in vivo decreases over time or that degradation products become demonstrable. Our group, however, failed to confirm either assumption [71]. Using the Western blot technique we were able to identify a single 32 kDa band at all time points after l-ASP infusion up to 24 hours (Fig. 5). Thus, we believe that degradation of the drug does not occur within the extracellular compartment.

B. Pharmacokinetics of PEG-l-ASP

Like in animals, PEGylation markedly prolongs half-life of l-ASP in humans. An *Achromobacter* preparation showed an increase from < 30 to 72 hours [72]. Using

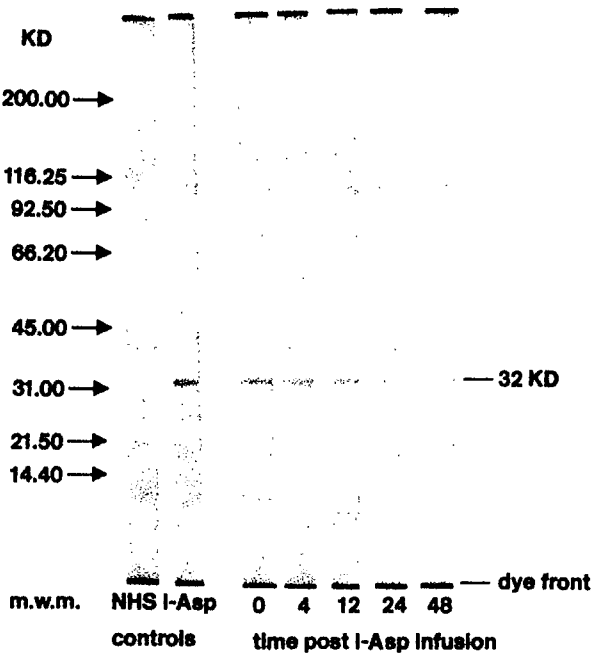

Figure 5 Western blot of l-Asp detected in serum of a patient at various time points after l-Asp administration. Only a single band at 32 kDa is detectable up to 24 h after l-Asp infusion. Neither a change of the molecular weight nor newly generated bands can be observed. m.w.m. = Molecular weight markers; NHS = normal human serum.

the *E. coli* enzyme, Keating et al. [73] calculated $t_{1/2}$ as 357 hours for the PEGylated enzyme compared to 20 hours for the conventional drug. It seems noteworthy to mention that all 37 patients studied had refractory malignancies and had experienced heavy pretreatment, including l-ASP. l-Asparagine depletion persisted until the next dose of PEG-l-ASP was given 2 weeks later.

C. Efficacy of Native Enzyme

Soon after the discovery of l-ASP as an antineoplastic agent, its antitumor specificity was studied in patients with cancer. Unfortunately, only one tumor consistently responded to the drug: childhood and adult ALL. Other tumors responded inconsistently, like acute myeloblastic leukemia (AML), non-Hodgkin's

lymphoma (NHL), and chronic leukemias, while most of the solid tumors were resistent to the drug [74,75]. Laboratory observations of the sensitivity of T-cell–derived tumor cells compared to those of B-cell origin [76] have not been explored in clinical trials.

Several requirements must be fulfilled for enzymes to be used for therapy: large-scale availability, little acute or chronic toxicity, low glutaminase activity, reasonable in vivo half-life, high substrate affinity, high specific enzyme activity, oncolytic activity. As not all l-ASPs fulfill these requirements, only two preparations are currently used: *E. coli* and *Erwinia.*

One of the protocols that clearly shows the clinical importance of l-ASP for treatment of childhood ALL was published by Clavell et al. [77]. The authors used a four-agent induction regimen (prednisone, vincristine, methotrexate, doxorubicine) followed by multiple agent therapy including weekly *E. coli* l-ASP at 25,000 U/m^2 i.m. Results were excellent and have hardly been improved in subsequent studies.

It is hardly possible to summarize all the treatment protocols currently used for the treatment of ALL. Basically, most protocols employ the same drugs with some differences in dose and mode of application; no profoundly new ideas have been developed since 1986. Table 5 is certainly incomplete but indicates current concepts for multiple agent chemotherapy. All these protocols use l-ASP as part of induction therapy in order to induce remissions in both ALL and acute nonlymphoblastic leukemia (ANLL).

D. Efficacy of PEG-l-ASP

Information about the clinical usefulness of PEG-l-ASP is still limited. Yoshimoto et al. [29] treated three children with multiple relapses of ALL who had developed anaphylactic shocks following native l-ASP. Successive administrative of PEG-l-ASP was well tolerated and effective. Our group administered PEG-l-ASP at a dose of 2000 U/m^2 as a 2-hour infusion every 2 weeks to five children with second relapse in ALL. The enzyme was combined with prednisone, vincristine, adriamycin, and methotrexate (MTX) i.t. Toxicity was transient and mild. Remission was achieved in five of five children lasting from 3 to 9 months [30]. Muss et al. [94] administered PEG-l-ASP at the same dose to 21 adult patients with refractory non-Hodgkin's lymphoma. Only 2 of 21 patients showed partial responses. Eleven of 21 had to be removed from the study due to disease progression and the remaining 9 due to toxicity. Another 37 heavily pretreated patients with refractory hematological malignancies were reported [95]. The doses used were variable. Complete remission was achieved in 2 patients with lymphoma and one with ALL. In a larger trial in children [96,97], 42 patients with ALL or acute undifferentiated leukemia (AUL) in relapse were

Table 5 Design of Chemotherapy Protocols Currently Used to Treat Adult or Childhood ALL and ANLL

Leukemia (untreated + relapse)	Concomitant medication (other than l-ASP)				Authors (Ref.)	Year of publication	Country
	Pred	VCR	CNS proph.	Others			
ALL (C)	+	+	+	MTX, 6-MP	Eden et al. (78)	1987	UK
AML (A)				HIDAC	Radtke et al. (79)	1988	Germany
ALL (C)	+	+	+	MTX, 6-MP	Van Eys et al. (80)	1989	USA
ALL (C)	+	+	+	MTX, 6-MP, Rubidomycin	Van der Does et al. (81)	1989	Holland
ALL (A)	+	+	+	Doxorubicin	Radford et al. (82)	1989	USA
ALL (C)	+	+	+	MTX, 6-MP, Ara-C, immunotherapy	Komada et al. (83)	1990	Japan
ALL (C)	+	+	+	MTX, Ara-C, VM 26, 6-MP, Daunorubicin	Henze et al. (84)	1990	Germany
ANLL (C)				MTX	Hudson, et al. (84)	1990	USA
ANLL (A)				HIDAC	Evans et al. (86)	1990	USA
ALL (C)	+	+		Daunomycin	Gaynon et al. (87)	1990	USA
ALL (C)				MTX, Ara-C	Gruemayer et al. (88)	1990	Austria
ALL (C)	+	+	+	Ara-C, Daunomycin, Teniposide	Belasco et al. (89)	1990	USA
ALL (A)	+	+		Ara-C, Daunomycin, 6-MP, Cyc, Adriamycin, Dexa	Lluesma et al. (90)	1991	Argentina
ALL (A)	+	+	+	MTX, Ara-C, Cyc, Adriamycin, 6-MP	Jacobs and Wood (92)	1992	South Africa
ALL (C)	+	+	+	MTX, Dexa	Tsukada et al. (93)	1993	Japan

A = Adults; C = children; MTX = methotrexate; MP = mercaptopurine; HIDAC = high-dose cytosin-arabinoside; Ara-C = cytosine-arabinoside; Cyc = cyclophosphamide; Dexa = dexamethasone.

enrolled. PEG-l-ASP was used in combination with vincristine, prednisone, and doxorubicine. All patients had been pretreated with conventional l-ASP. Following a 2-week monotherapy period, 22% of patients went into partial or complete remission. Toxicity including allergic reactions was mild and appeared to be milder than expected from historical data.

VI. TOXICITY AND IMMUNOGENICITY

Several side effects have been described in connection with l-ASP therapy. A summary is given in Table 6 [6]. The frequency seems to depend on the type of preparation used [58] (Table 7), although these data have not been obtained in trolled trials.

A. Toxicity

1. The Clotting System

a. Thrombosis

Both thrombotic and hemorrhage complications have been described in 1–5% of patients under l-ASP therapy. Gugliotta et al. [98] performed a retrospective survey in 238 adult ALL patients who had received l-ASP as part of multiple agent chemotherapy. Ten patients had developed thrombotic complications, five of which were lethal. Thrombosis mostly affected the cerebral sinus. Other vessels were cerebral arteries, portal vein, pulmonary district, and a deep vein of a lower extremity. An increased risk for such events seemed to be indicated by an

Table 6 Summary of Adverse Effects Observed in Association with l-ASP Therapy

System	Adverse effects
Immune	Hypersensitivity, urticaria, anaphylactic shock
Pancreas	Acute hemorrhagic pancreatitis; pancreatitis, with decreased serum insulin; diabetic ketosis
Liver	Fall in serum albumin; rise in alkaline phosphatase; increase in bromsulphalein retention; increase in serum bilirubin and SGOT; decrease in serum cholesterol; decrease in α- and β-lipoprotein
Coagulation	Increase in prothrombin time; decreases in fibrinogen, plasminogen, factors V, VII, VIII, IX and others; thrombotic and hemorrhagic events
CNS	Mild depression and personality changes; confusion and hallucinations

Source: Modified from Ref. 6.

Table 7 Grade 3/4 Toxicities of l-ASP Preparations

Reaction	*E. coli*	*Erwinia*	PEG-l-ASP
Allergic reaction	11	2	5
Hyperglycemia (requiring insulin)	4	1	2
Pancreatitis, elevated amylase	2	0	3
Hypofibrinogenemia (grade 4)	2	1	3
Coagulopathy (grade 4)	3	3	1
Neurological disorder	3	2	2

Data represent percent reacting patients.
Source: Modified from Ref. 53.

increase in fibrinopeptide A, thrombin-antithrombin complexes, and prothrombin fragments F1 + 2 [99].

One anecdotal report exists about a child with ALL developing vertebral osteonecrosis probably secondary to thrombosis while receiving l-ASP therapy [100]. Another report describes a myocardial infarction [101].

Many groups have studied changes in the coagulation system induced by the enzyme. Decreased plasma levels of antithrombin III (AT III) [102–105], plasminogen [102], or protein C [103,104,106,107] and protein S [104,106] were described as the cause of thrombotic complications, but other investigators failed to confirm these results [108,109]. O'Meara et al. [110] even showed that the AT III antigen steadily increased throughout the study period while AT III function remained unchanged. Pui et al. [111] analyzed von Willebrand factor (vWF) by SDS agarose gel electrophoresis. Their results suggested that thrombotic complications resulted from platelet agglutination by plasma vWF. The last study seems to be of special importance because the authors studied patients who in fact experienced thrombotic events while the others described changes in the clotting system in asymptomatic patients. For comparative purposes the different treatment modalities used in the different report have to be taken into account as a possible explanation for the inhomogeneous results. The origin of the hypercoagulable state induced by l-ASP remains obscure. Semerano [112] found procoagulant activity of peripheral blood mononuclear cells significantly higher in children with ALL during the period of l-ASP treatment. Plasma D-dimers were increased at the same time. Type 1 plasminogen activator inhibitor was markedly elevated, whereas plasminogen decreased. Taken together these changes may promote fibrin formation. Hemostaseological changes induced by *Erwinia* asparaginase seem to be less than those with the *E. coli* product [113], however, thrombotic events have occurred despite these findings [114].

Some attempts have been made to correct low AT III levels by i.v. infusion [115]. Satisfactory AT III levels can be maintained during l-ASP treatment by a commercially available preparation. In another study the authors showed that following replacement of AT III the enhanced thrombin generation, D-dimer formation, and plasminogen activator inhibitor 1 were reduced to normal levels, indicating disappearance of markers of hypercoagulation [116]. However, whether or not this procedure not only improves hemostaseology but also prevents thrombotic events remains to be established. Fresh frozen plasma does not seem to able to correct AT III deficiency induced by l-ASP treatment [117].

Treatment of manifest thrombosis under l-ASP is not standardized. Castaman et al. [118] were successful in one of two cases using a combination of fresh frozen plasma and s.c. heparin. Data on urokinase or recombinant tissue plasminogen activator (r-TPA) are unavailable to date.

b. Hemorrhage

Hemorrhagic complications are generally believed to result from decreased synthesis of clotting factors like fibrinogen, vitamin K–dependent factors, and independent factors [119]. Only a few factors remain unchanged.

Ott et al. [120] analyzed the impact of hemostaseological complications on long-term sequelae in 34 children with ALL. One child died as a consequence of a CNS event; another experienced autoamputation of a toe due to dorsal pedal artery occlusion. The other complications were transient and reversible.

2. Pancreas and Salivary Glands

Pancreatitis is a significant problem in patients undergoing l-ASP therapy. The reported incidence ranges from 8 to 18%, the mortality from 1.8 to 4.6% [121–123]. It may develop up to 16 weeks after treatment. Rarely, pancreatic pseudocysts have been reported.

Monitoring of patients is advised with respect to clinical symptoms (nausea, vomiting, abdominal pain) and abnormal laboratory values (elevated serum or urinary amylase, serum lipase). The diagnosis can be confirmed by ultrasonography [124], a method that is useless if solely applied as a screening method before clinical or laboratory evidence for pancreatitis has been obtained [125].

If islet cells are damaged, transient diabetes may occur. Some evidence exists that l-ASP–induced hyperglycemia can be attenuated after substitution of the *Erwinia* enzyme for the *E. coli* preparation [126]. This observation, however, is anecdotal. Whether or not the incidence of pancreatitis (diabetes) can successfully be reduced by switching from one preparation to the other can only be determined in randomized prospective trials.

Parotid glands may present with parotitis following treatment with l-ASP [127]. In such rare cases discontinuation of the enzyme seems to be advised.

3. Lipid Metabolism

Changes in serum lipids may be induced not only by l-ASP but also be pancreatitis and/or steroids. Cremer et al. [128] differentiated these effects in children with ALL. An increase of cholesterol, mainly α-cholesterol, could not be attributed to the action of l-ASP. In contrast, high levels of exogenous chylomicron-bound triglycerides were linked to l-ASP administration. All changes were fully reversible.

4. Endocrine Abnormalities

Reduced synthesis of proteins in the liver can also affect the endocrinium. Thyroxine-binding globulin (TBG), the major thyroid hormone transport protein, has been found to be transiently but markedly decreased during l-ASP therapy [129,130]. In extended observations Ferster et al. [131] demonstrated a decrease of total T4, FT4, total T3, and TBG during induction therapy. The changes in T4 and FT4 were attributable to l-ASP, while low T3 and TBG were due to glucocorticoid administration.

Effects similar to TBG were shown for sex-hormone–binding globulin (SHBG) [132]. The clinical relevance of this observation remains to be established.

5. Teratogenicity

l-ASP induced a panel of malformations in rat embryos [133], making the drug potentially teratogenic.

B. Immunogenicity

As bacteria-derived proteins are foreign to the human immune system, it is not surprising that their parenteral administration is followed by an immune response. This has been shown in humans soon after the discovery of the enzyme's oncolytic potential [134–136] and was confirmed several times afterwards [58,59,69,137–139].

Our first studies were published in 1983 [69]. We were able to demonstrate induction of both IgM and IgG antibodies to *E. coli* l-ASP that markedly exceeded the normal range after three i.v. infusions of the enzymes at 3000 U/kg bw every third day. High levels of specific antibodies were associated with an increased risk of allergic reactions. Antibody titers decreased upon

l-ASP infusion, indicating incorporation in circulating immune complexes (CIC). The consequences for pharmacokinetics have already been discussed above.

The question arose whether or not enzyme activity was affected following binding of the antibody. For this purpose IgG fractions of an experimental rabbit antibody to l-ASP were prepared and mixed with the enzyme at slight antigen excess. CIC separated from free l-ASP by gel filtration were still enzymatically active (Fig. 6), indicating that at least the rabbit antibody does not recognize the active site of the enzyme as an immunogenic epitope. Thus, if l-ASP activity decreases in the presence of antibodies, it is not due to the blockade of the active site but rather accelerated elimination.

Binding of IgG or IgM antibodies to antigen does not lead to clinically relevant events unless secondary reactions are triggered. The system most likely to be involved is the complement system. In our studies from 1985 [139], we were able to show three things:

1. Elevated IgG antibodies to l-ASP are associated with an increased risk of anaphylaxis upon l-ASP infusion ($p < 0.0001$; Fig. 7). This observation has been confirmed by others [140].
2. l-ASP infusions associated with anaphylaxis are in parallel associated with activation of the classical complement pathway (Fig. 8).
3. If no changes during infusions but only postinfusion values are monitored, C3d determined in EDTA-plasma is the best parameter to retrospectively confirm involvement of the complement system (Fig. 9).

In addition to these immediate changes within the complement system, which occur within minutes, there are "chronic" changes probably arising from hyposynthesis of the components as it has been discussed for the clotting factors. IgG antibodies predominantly belong to subclasses IgG1 and IgG3 [141], which coincides well with these subclasses' ability to activate the classical complement pathway.

A complement-independent mechanism of anaphylaxis would be classical allergy employing IgE antibodies. We found these antibodies to be elevated in only a few cases—possibly atopic individuals [142]. The serum of one of these children was used for passive sensitization of basophils of a healthy blood donor. The cells released histamine upon incubation of the cells with l-ASP (Fig. 10), indicating that in some children IgE-mediated allergy may also play a role for clinical symptoms.

Botana et al. [143] analyzed possible nonimmunological mechanisms involved in the release of anaphylactic mediators like histamine using rat mast cells. They were able to show that there was some release upon incubation of

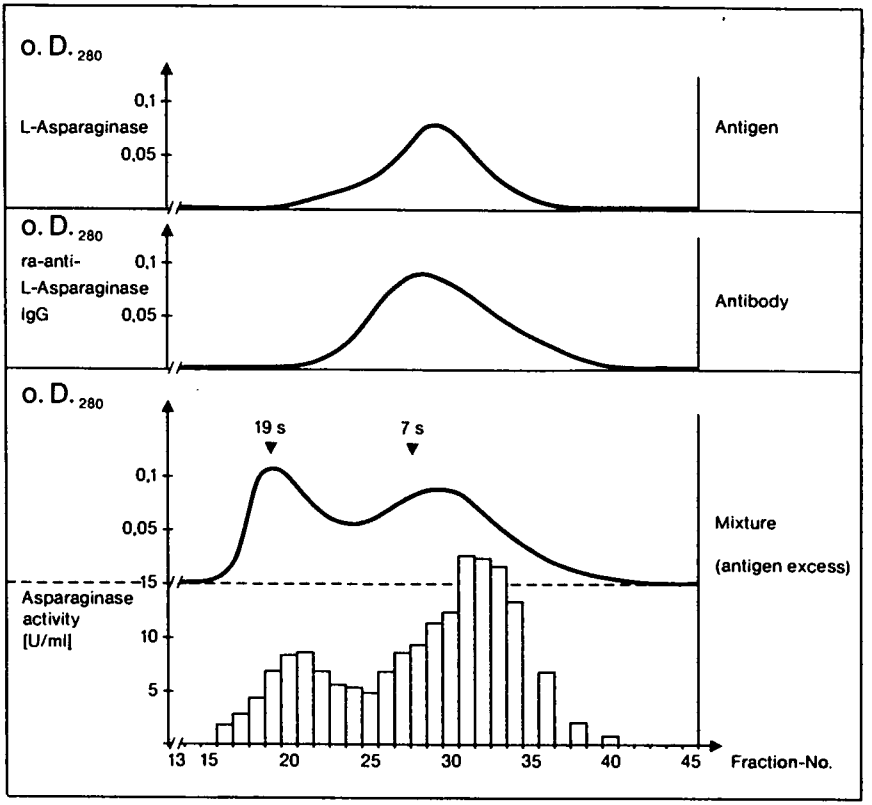

Figure 6 Demonstration of enzymatic activity in l-Asp–containing immune complexes. The upper panel shows the elution profile on a Sephadex G-200 column of l-Asp alone. All protein is eluted as a single peak. The second panel (from top) shows the elution profile of the IgC fraction of an experimental anti-l-Asp antibody. It is eluted from the same column as a single peak. The mixture of antigen and antibody is eluted in two peaks (panel 3) where the 19 s peak most likely represents soluble immune complexes. The assay for l-Asp in these fractions shows that soluble complexes are still enzymatically active (lower paths).

cells with l-ASP, but the extent was low compared to drugs like cytarabine, carboplatin, or etoposide. Upon purification or treatment of the cells with theophylline, their ability to release histamine is lost [144].

Erwinia l-ASP is sometimes claimed to be less immunogenic than the *E. coli* preparation [145–147], however, randomized prospective trials confirming this assumption have not been performed so far. The reason why *Erwinia* l-ASP might

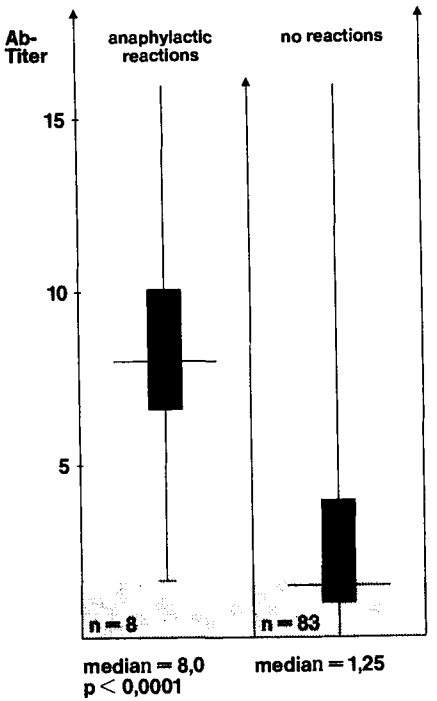

Figure 7 Comparison of IgG antibody levels preinfusions between infusions associated with anaphylaxis and those that were clinically tolerated (box plot). The difference is statistically significant but no absolute prediction of reactions is possible. The normal range is given by the shaded area.

induce a lower antibody response is that the human organism, due to the higher enzyme activity of the *Erwinia* enzyme, is challenged by a lower amount of antigen. Duration of exposure of the drug to the immune system does not differ significantly because in vivo half-lives are comparable in humans. Our own studies clearly show that *Erwinia* l-ASP is immunogenic in vivo (Fig. 11) (Wahn and Drewelius, unpublished). However, these data were obtained in a setting markedly different from the protocol where the *E. coli* l-ASP data were obtained.

Many groups now switch from one preparation to the other in order to avoid allergic reactions. Some of our studies support this concept:

1. An experimental rabbit antibody raised against *E. coli* l-ASP does not react with the *Erwinia* preparation on double diffusion (Fig. 12).

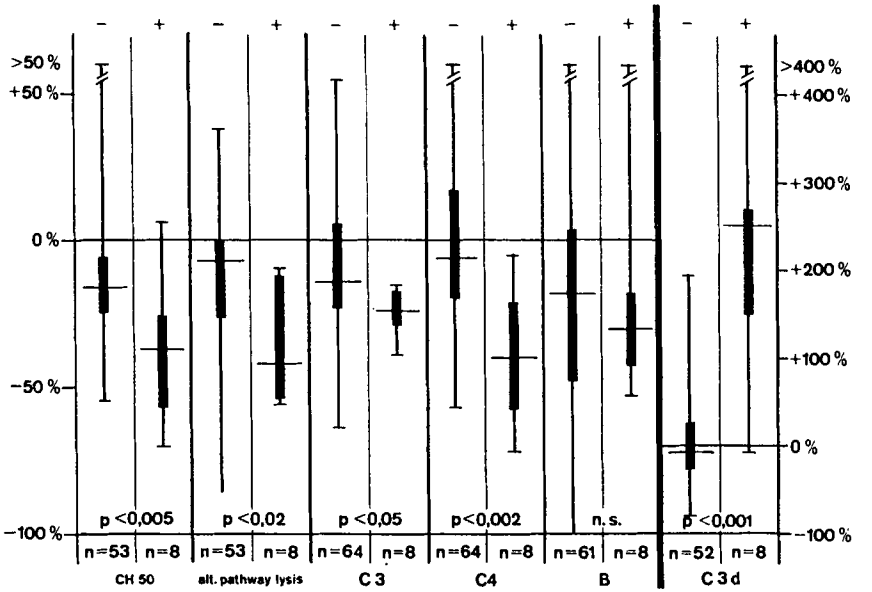

Figure 8 Comparison of changes in the complement system during l-Asp infusions between infusions that were clinically tolerated and those associated with anaphylaxis (box plot). All differences between the two groups are statistically significant except for those for factor B.

2. Patient's serum containing antibodies to *E. coli* l-ASP does not bind to solid phase *Erwinia* l-ASP (Fig. 13).
3. Absorption studies in the fluid phase using the principle of the RAST inhibition assay exclude cross-reactivity (Fig. 14) (Wahn and Drewelius, unpublished).

Unfortunately the still low number of available l-ASP preparations limits the number of possible switches. If one wants to take advantage of l-ASP's oncolytic potential over a longer period of time, new approaches are required. Concomitant infusion of epinephrine in parallel to l-ASP [148] may be helpful with respect to anaphylaxis but does not solve the basic problem of immunogenicity and altered pharmacokinetics.

Possibilities of using chemically modified enzymes with reduced immunogenicity have already been discussed above. However, before, for instance, PEG-l-ASP routinely enters clinical protocols, it must be clarified by randomized trials whether or not a G-phase–specific drug like l-ASP interferes with

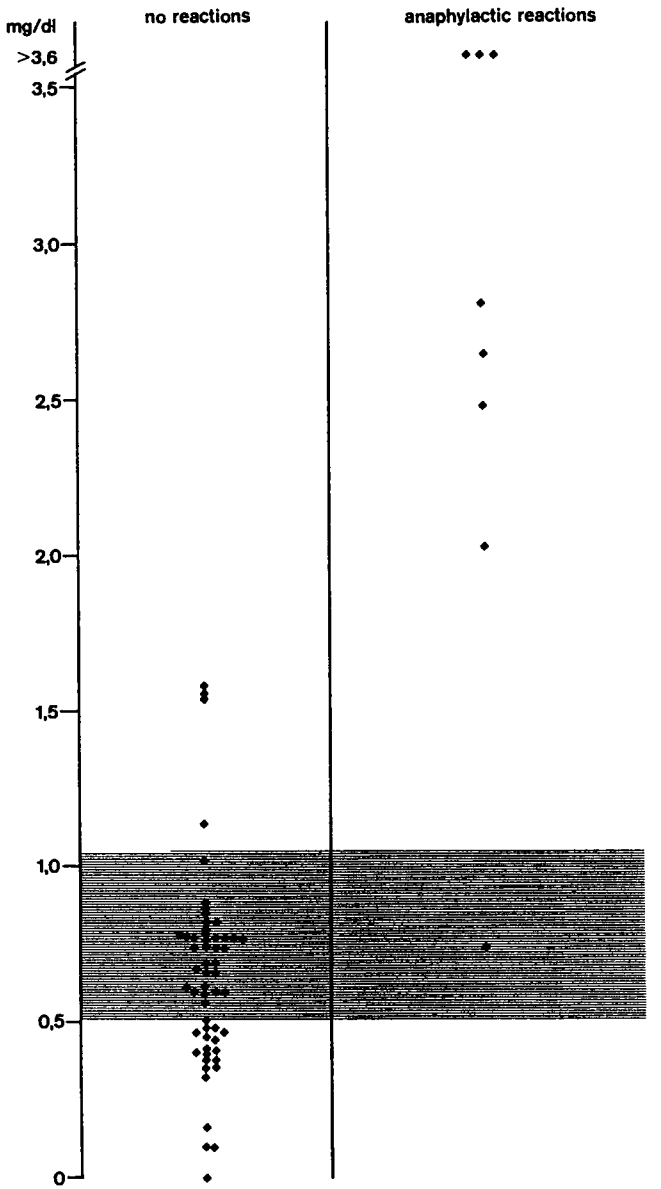

Figure 9 Levels of C3d in EDTA-plasma following l-Asp infusions. The normal range is indicated by the shaded area. Levels associated with clinical tolerance (left) are all lower than 1.7 mg/dL, levels associated with anaphylaxis (right) are all higher than 1.7 mg/dL except one. The difference is significant ($p < 0.0001$).

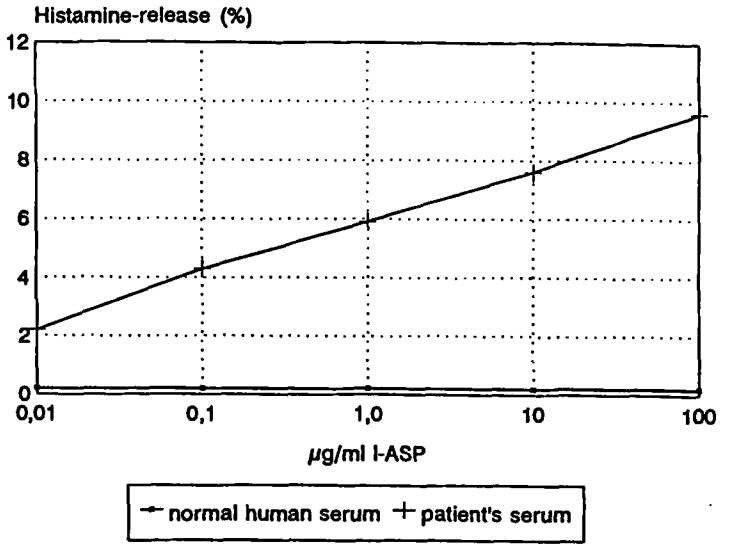

Figure 10 Demonstration of histamine release of normal human basophils sensitized with either normal human serum (+) or serum of a child with clinical anaphylaxis to l-ASP (+) following exposure to increasing concentrations of l-ASP. Data are expressed as percent of releasable histamine.

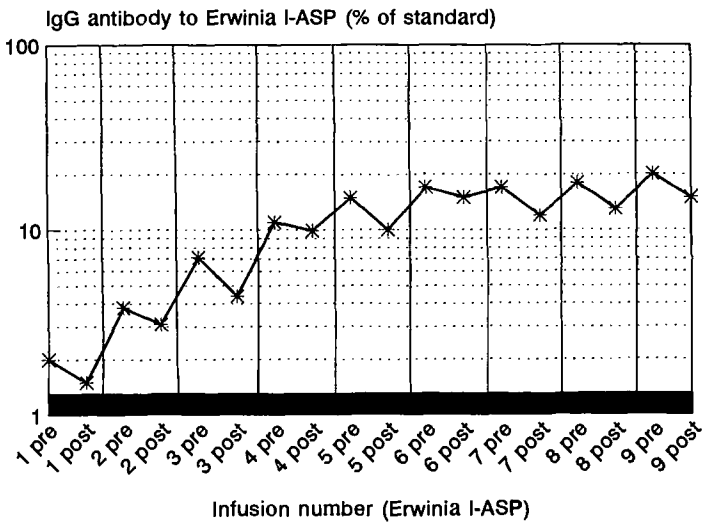

Figure 11 Development of IgG antibodies to *Erwinia* l-ASP in children treated with this drug for ALL. Medians of values obtained either pre- or postinfusions are plotted. Antibody production is clearly stimulated with consistent consumption of antibodies during infusions.

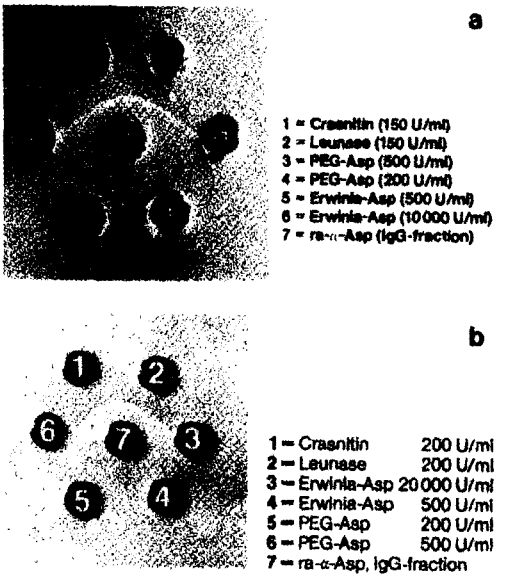
a
1 = Crasnitin (150 U/ml)
2 = Leunase (150 U/ml)
3 = PEG-Asp (500 U/ml)
4 = PEG-Asp (200 U/ml)
5 = Erwinia-Asp (500 U/ml)
6 = Erwinia-Asp (10000 U/ml)
7 = ra-α-Asp (IgG-fraction)
b
1 = Crasnitin 200 U/ml
2 = Leunase 200 U/ml
3 = Erwinia-Asp 20000 U/ml
4 = Erwinia-Asp 500 U/ml
5 = PEG-Asp 200 U/ml
6 = PEG-Asp 500 U/ml
7 = ra-α-Asp, IgG-fraction

Figure 12 Double diffusion experiments using a polyclonal rabbit antibody to the *E. coli* l-ASP (center well) against four different l-ASP preparations. Only the unmodified *E. coli* preparation forms a precipitin line of complete identity (a), whereas after freezing and thawing PEG-ASP also reacts (b).

S-phase–specific drugs like adriamycin or M-phase–specific drugs like vincristine. If these possible hazards can be ruled out, a drug like PEG-l-ASP may represent a significant step ahead toward better tolerance of the enzyme.

VII. PROSPECTS FOR THE FUTURE

An individualized approach toward treatment only of patients whose blasts are sensitive to l-ASP seems to be reasonable but is difficult to perform especially if their numbers are low and inaccessible for biochemical studies. As a feasible alternative, the expression of l-asparagine synthetase mRNA should be monitored in bone marrow aspirates and correlated to the overall treatment response.

The whole potential of l-ASP has probably not been fully elucidated due to its treatment-limiting immunogenicity. Two major ways might be able to

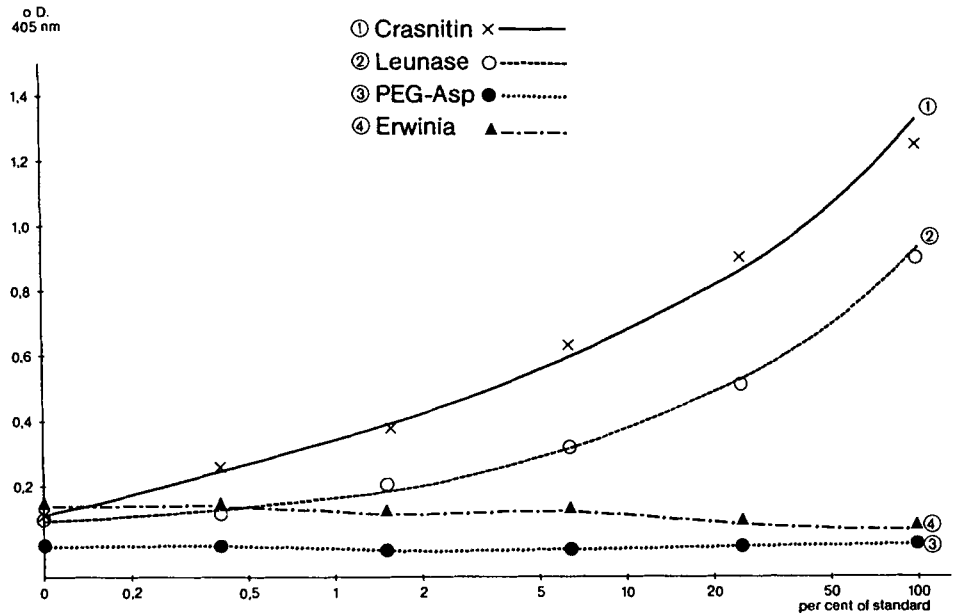

Figure 13 Relativity of four different l-Asp preparations immobilized on a microtiter with human antibodies to *E. coli* preparation I. It is expressed as o.d. 405 nm values. The patient's serum (standard serum) was diluted serially in pooled normal human serum before antibodies were quantified by ELISA. The antibodies bind significantly better to both *E. coli* preparations compared to *Erwinia*- and PEG-Asp.

reduce this problem. One is to reduce the drug's immunogenicity by chemical modification of relevant epitopes. The other would be to induce "real" tolerance, i.e., to activate immune mechanisms involved in immune tolerance. That this is possible via the oral route has been shown in animal model for type II collagen-induced arthritis [149] as well as in humans with rheumatoid arthritis [150]. My personal hope remains that details for such an approach will be worked out in the future and will lead to an even more effective mode of l-ASP administration.

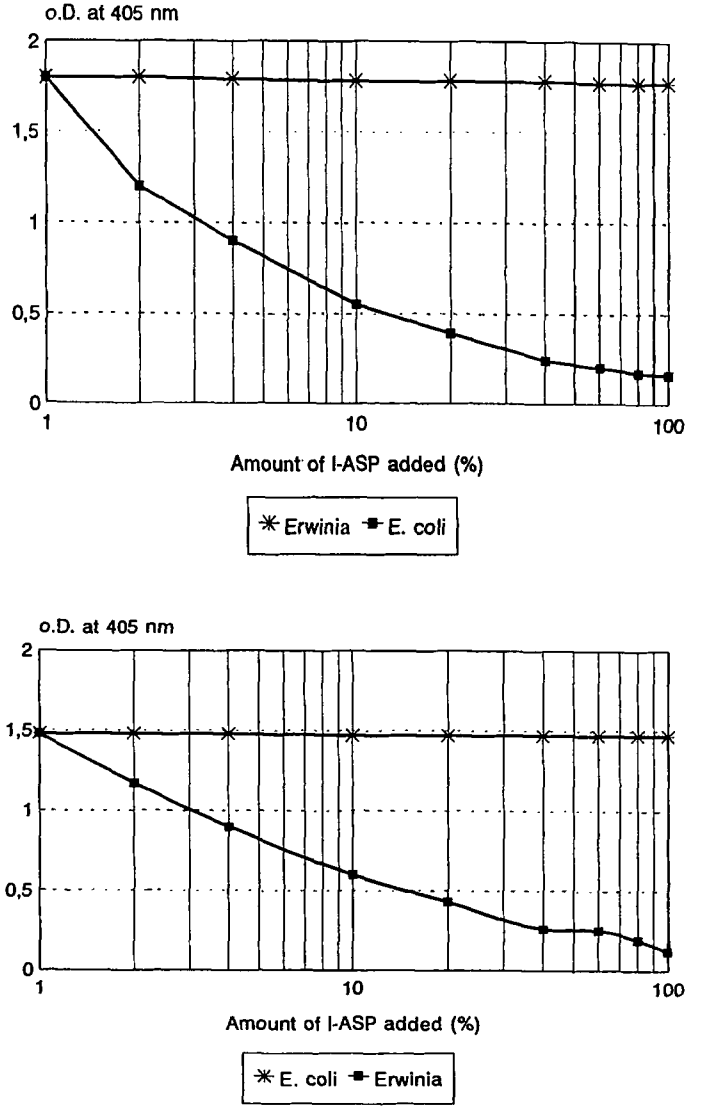

Figure 14 Absence of cross-reactivity between *E. coli* and *Erwinia* l-ASP shown by solid phase EIA. Plates were coated with either *E. coli* l-ASP (upper panel) or *Erwinia* l-ASP (lower panel). In the upper panel a serum of a patient with high titers of antibodies exclusively to *E. coli* l-ASP was used, in the lower panel serum of a patient with high titers of antibodies exclusively to *Erwinia* l-ASP. In the upper panel only *E. coli* l-ASP is able to absorb out antibody activity, while in the lower panel only *Erwinia* l-ASP shows this behavior.

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11

Macrophage-Targeted Glucocerebrosidase: A Therapeutically Effective Enzyme Replacement Product for Gaucher Disease

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I. INTRODUCTION

Gaucher disease is the most prevalent hereditary metabolic storage disorder of humans [1,2]. It is caused by a deficiency of lysosomal β-glucocerebrosidase activity [3,4]. Glucocerebroside, the naturally occurring glycolipid substrate of this enzyme, progressively accumulates within cells of the reticuloendothelial system and produces distinctive lipid-laden macrophages in multiple organs. The clinical manifestations of the disorder are well known and include anemia, coagulation abnormalities, hepatosplenomegaly, and structural skeletal changes. Patients with these findings who do not exhibit signs of central nervous system involvement are classified as having type 1 Gaucher disease. This is the most prevalent form of the illness. Type 2 Gaucher disease is a rare phenotype that presents during infancy and is characterized by hepatosplenomegaly and rapidly progressive brain involvement [5]. Type 3 Gaucher disease is another infrequently encountered phenotype in which slowly progressive central nervous system disease occurs in conjunction with the systemic symptoms typical of type 1 disease [6].

Shortly after the discovery of glucocerebrosidase deficiency in Gaucher disease, several strategies were proposed for the treatment of patients with this disorder [7]. Among the proposals, enzyme replacement was a particularly attractive possibility. The likelihood that exogenous enzyme might have to be targeted to a specific cell type was also put forth at that time. Several decades of clinical and laboratory research were required to bring enzyme replacement therapy to its present state of development as the dominant therapeutic modality for Gaucher disease. Salient events that culminated in this achievement are cited here and logical extensions of this accomplishment are indicated. The lessons learned during these investigations are likely to be applicable to the development of effective enzyme therapy in a number of other metabolic disorders of humans.

II. NASCENT INVESTIGATIONS

In order to lessen the possibility of sensitizing patients to an exogenous protein from a nonhuman source, human placental tissue was selected as a potential source of glucocerebrosidase. Since both fresh and frozen placental extracts exhibited significant glucocerebrosidase activity, an investigation was undertaken to isolate the enzyme from this tissue. Glucocerebrosidase was difficult to purify using the conventional procedures known at the time, because it was found to be a highly hydrophobic protein. These difficulties were surmounted by including a nondenaturing detergent in each of the steps used to purify the enzyme. Small amounts of highly purified glucocerebrosidase were eventually obtained with these modifications [8]. We used these preparations to examine several important aspects of enzyme replacement. We first learned that the intravenous injection of exogenous glucocerebrosidase was well tolerated by patients with Gaucher disease. Second, we discovered that the quantity of glucocerebroside in liver was lower after a small amount of the enzyme was infused into patients with Gaucher disease [9]. This important discovery set the course for subsequent efforts that culminated in the demonstration of the clinical effectiveness of enzyme replacement therapy. Without the demonstration that injections of glucocerebrosidase caused a reduction of accumulated glucocerebroside, it is likely that enzyme replacement therapy for Gaucher disease would not have been pursued for many years, if ever. The details regarding the amounts of enzyme injected and the quantitative responses to it have been published elsewhere [10].

These initial investigations suggested that larger quantities of glucocerebrosidase, administered repeatedly, would be necessary in order to expect clinical benefit. Because we were unable to scale up the original procedure used to isolate glucocerebrosidase, we expended considerable effort to develop a novel purification strategy using hydrophobic column chromatography to take advantage of the high degree of hydrophobicity of glucocerebrosidase [11]. This technique

provided glucocerebrosidase of high purity and in good yield. However, when we injected this enzyme into patients with Gaucher disease, we did not obtain consistent reductions of hepatic glucocerebroside (Table 1).

We were initially unable to explain these variations in response. We had shown that the enzyme was fully active catalytically using ^{14}C-glucocerebroside as substrate. We wondered whether an inhibitor might have been present in some of the biopsy specimens. In order to dispel this possibility, we incubated glucocerebrosidase purified by hydrophobic column chromatography with liver biopsy specimens obtained from patients with Gaucher disease. We found that the addition of enzyme to these samples in vitro brought about the catabolism of all of the accumulated glucocerebroside [12]. This demonstration was another major step forward in the development of successful enzyme replacement therapy for Gaucher disease, since it was conceivable that the storage material might have been present in a physical state that was completely refractory to the catalytic activity of the enzyme. Moreover, it indicated that co-administration of supplemental factors such as saposin C, a protein co-hydrolase, or an anionic lipid such as phosphatidylserine, was not required for the catabolism of glucocerebroside in vivo, although these factors are necessary ingredients when the catalytic activity of glucocerebrosidase is measured in vitro in the absence of detergents. It is hard to overemphasize the significance of this finding, since a requirement for the simultaneous administration of such additional agents would have greatly increased the complexity of enzyme replacement therapy. These experiments strongly suggested that the inconsistencies observed in the effects of exogenous glucocerebrosidase were due to variable delivery of the enzyme to lipid-storing macrophages such as the Kupffer cells in the liver.

Table 1 Inconsistent Effect of Intravenous Infusions of 1000 U Glucocerebrosidase Purified by Hydrophobic Chromatography on the Amount of Glucocerebroside in the Liver of Patients with Gaucher Disease

Patient		Glucocerebroside (μg/g liver)[a]		
Age (y)	Sex	Preinfusion	24 Hours postinfusion	% Change
30	F	1500	300	–80
36	M	3100	3100	0
56	F	3200	3200	0
33	M	14000	14000	0
32	F	15000	15000	0
35	F	25000	21000	–16
37	F	26000	13000	–50

[a]Normal range: 45–90 μg/g liver.

III. CARBOHYDRATE MODIFICATION OF GLUCOCEREBROSIDASE

We performed a series of investigations in experimental animals in which it was discovered that only a small proportion of intravenously injected glucocerebrosidase prepared by the hydrophobic chromatography procedure actually reached Kupffer cells in the liver [13]. Development of a targeting strategy that could be used to deliver useful quantities of glucocerebrosidase to macrophages was dependent upon two independent observations. Goldstone and Koenig discovered that lysosomal enzymes were glycoproteins [14], and Stahl and coworkers found that macrophages expressed a lectin on their surface that interacts avidly with mannose-terminal glycoconjugates [15]. We analyzed the carbohydrate composition of native human placental glucocerebrosidase and found that it contained four oligosaccharide chains [16]. Three of these side chains were of the complex type and terminated for the most part with sialic acid molecules (Fig. 1). However, some of the oligosaccharide chains were not sialylated and had terminal molecules of galactose. Intravenous injection of the native, fully glycosylated enzyme into rats revealed that approximately 96% was taken up by hepatocytes, probably because of a lectin on these cells that has a high affinity for galactose-terminal glycoproteins [17]. Since significant quantities of glucocerebroside do not accumulate in hepatocytes, probably as a result of biliary excretion of this lipid [18], the likelihood of clinically beneficial effects following infusion of the native enzyme preparation appeared to be slight.

In order to direct exogenous glucocerebrosidase to macrophages, we decided to utilize the mannose lectin on the surface of these cells. In an initial experiment, linear pentamannosyl chains were covalently attached to the enzyme [19]. The delivery of glucocerebrosidase to macrophages was not increased by this modification. We next covalently linked trimannosyl-dilysyl residues to glucocerebrosidase [20]. Although this modification resulted in a fourfold increase in the delivery of glucocerebrosidase to Kupffer cells, we felt that this increase would not be sufficient to provide consistent clinical benefit. Eventually, the internal mannose residues of native placental glucocerebrosidase were

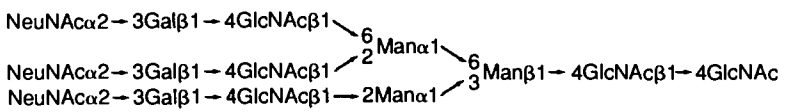

Figure 1 Carbohydrate composition of the predominant oligosaccharide chain of human placental glucocerebrosidase. Gal = Galactose; GlcNAc = N-acetylglucosamine; Man = mannose; NeuNac = N-acetylneuraminic acid.

exposed by sequential deglycosylation of the protein with exoglycosidases (Fig. 2) [21,22]. With this modification, a striking increase in the delivery of glucocerebrosidase to hepatic nonparenchymal cells, including Kupffer cells, was observed (Fig. 3). Although the specificity of this process has been challenged [23], recent immunoelectronmicroscopic studies demonstrate highly selective localization of mannose-terminal glucocerebrosidase within the lysosomes of Kupffer cells (Fig. 4) and, to a lesser extent, sinusoidal endothelial cells [24,25].

Encouraged by the greatly enhanced uptake of macrophage-targeted glucocerebrosidase by reticuloendothelial cells, we initiated an investigation to assess the clinical effects of this neoglycoform of glucocerebrosidase.

IV. FIRST DEMONSTRATION OF CLINICAL RESPONSES TO MACROPHAGE-TARGETED GLUCOCEREBROSIDASE

The clinical effects of mannose-terminal glucocerebrosidase were initially examined in eight patients with non-neuronopathic, type 1 Gaucher disease. Seven of the patients were either adolescents or adults, and one was a 4-year-old boy. A total dose of 190 U of enzyme was administered intravenously to each of these patients weekly over a 6-month period. A beneficial effect was clearly evident only in the youngest of the recipients [26]. A dramatic increase in

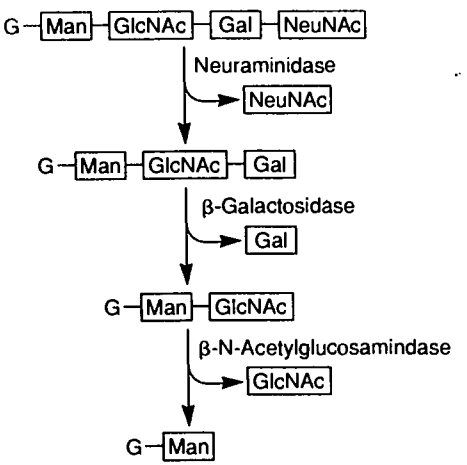

Figure 2 Production of mannose-terminal glucocerebrosidase by sequential deglycosylation of the native placental enzyme with exoglycosidases. Abbreviations as in Figure 1. (From Ref. 10.)

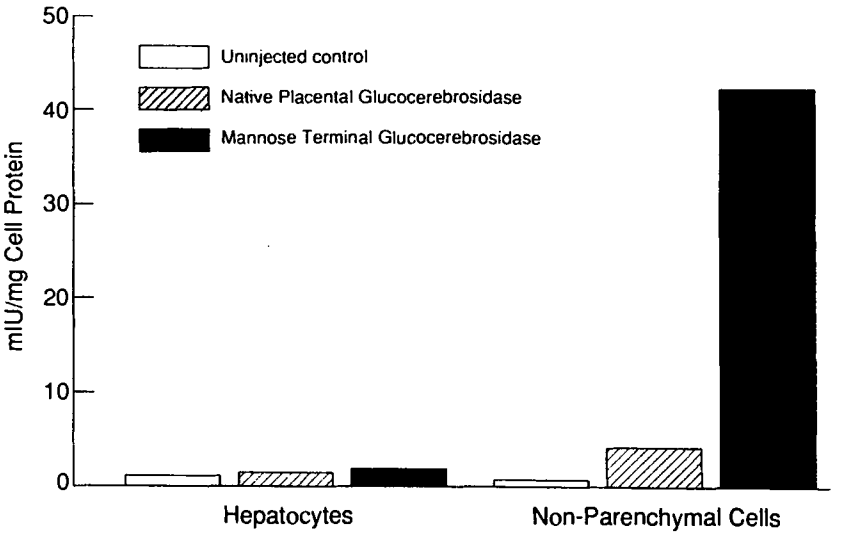

Figure 3 Bar graph showing uptake of glucocerebrosidase by hepatocytes and non-parenchymal cells isolated from rat liver after infusion of 6.7 U of native or 5.0 U of mannose-terminal enzyme. (Adapted from Ref. 22.)

hemoglobin from severely anemic values to a nearly normal level occurred in this patient after several months of treatment with macrophage-targeted glucocerebrosidase at a dose ranging from 9 to 12 U/kg body weight. The platelet count also increased significantly. Enzyme infusions were then discontinued to determine whether these changes might be attributed to a spontaneous remission of the disease. Hemoglobin and platelet levels gradually declined to the preinfusion values over a 6-month interval during which the patient received no enzyme. We felt that this child responded to macrophage-targeted glucocerebrosidase because he had received considerably more enzyme per kilogram of body weight than any of the other participants in the trial.

Glucocerebrosidase injections were then resumed in this patient, but at a dose of 30 U/kg body weight each week. In comparison with the responses observed at a dose of 9–12 U/kg body weight, the hemoglobin rose at a more rapid rate with the higher dose infusions and ultimately returned to a fully normal value (Fig. 5). In addition, serum acid phosphatase (Fig. 6) and plasma glucocerebroside (Fig. 7) levels and the degree of hepatosplenomegaly (Fig. 8) decreased substantially. The Gaucher cell infiltrate was also cleared from the

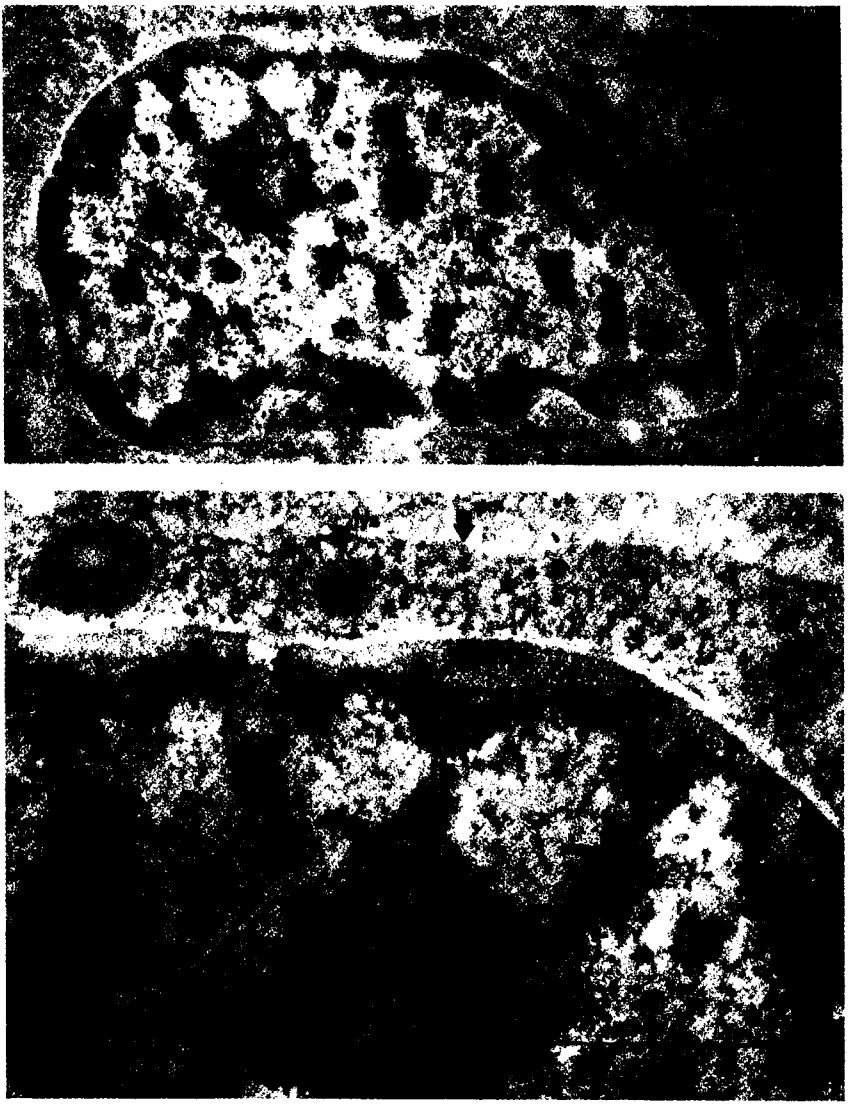

Figure 4 Immunogold localization of mannose-terminal glucocerebrosidase in rat liver Kupffer cells 30 minutes after enzyme infusion. Gold particles are concentrated in the lysosomes. Lys = Lysosome; M = mitochondrion; Nuc = nucleus; PM = plasma membrane. Original magnifications: (A) ×10,000; (B) ×33,000. Bars = 1 μm. (From Ref. 24.)

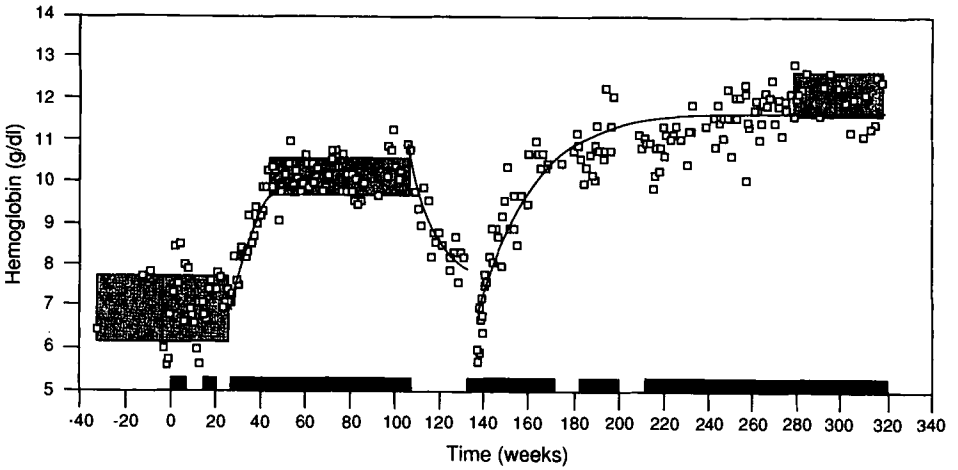

Figure 5 Effect of weekly intravenous infusions of mannose-terminal glucocerebrosidase on hemoglobin concentration in a 4-year-old boy with type 1 Gaucher disease. Solid black bars along the abscissa represent periods of enzyme treatment. A low dose of enzyme (9–12 U/kg) was administered between weeks 1 and 105; dosage was increased to 30 U/kg between weeks 131 and 320. Shaded areas represent the mean pretreatment and apparent steady-state values observed during the periods of low- and high-dose enzyme supplementation (±1 SD). Normal range: 11–15 g/dL. (From Ref. 10.)

bone marrow (Fig. 9), and there was evidence of skeletal improvement (Fig. 10) [27,28]. The more robust responses observed at 30 U/kg body weight suggested to us that the clinical responses to macrophage-targeted glucocerebrosidase were dose dependent.

V. DOSE-RESPONSE TRIAL

Because we assumed that the variations in clinical response observed in the preceding trial were related to the relative amount of enzyme each patient received, we conducted a detailed investigation of the pharmacokinetic and pharmacodynamic properties of macrophage-targeted glucocerebrosidase in 23 patients with type 1 Gaucher disease who had intact spleens [29]. Each patient received a single infusion of enzyme over 4 hours; a dose range of 0.6–234 U/kg body weight was examined. Steady-state enzymatic activity was achieved in plasma within 60 minutes. When the infusion was terminated, macrophage-targeted glucocerebrosidase was eliminated from the plasma compartment by a

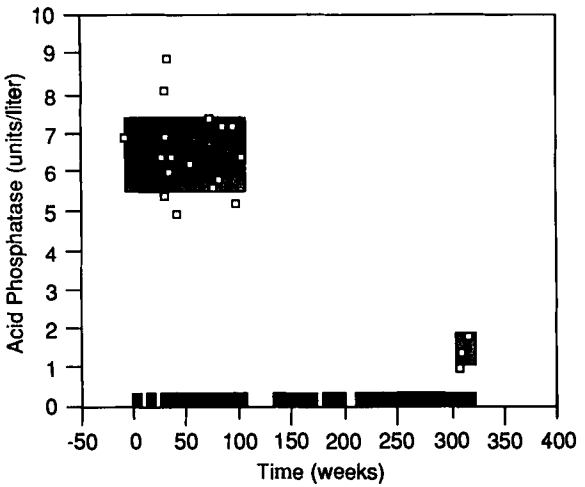

Figure 6 Effect of weekly intravenous infusions of mannose-terminal glucocerebrosidase on serum acid phosphatase activity. Enzyme was administered as described in Figure 5. Shaded areas represent the mean values observed during the entire low-dose period and after 2 years of uninterrupted high-dose treatment (±1 SD). Normal range: 0–0.4 U/L.

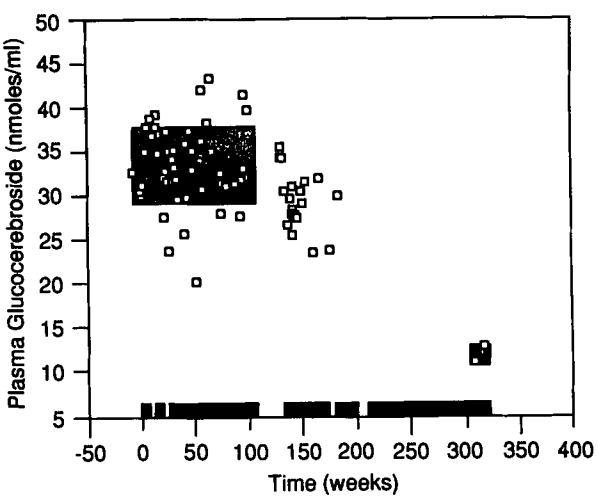

Figure 7 Effect of weekly intravenous infusions of mannose-terminal glucocerebrosidase on plasma glucocerebroside levels. Enzyme was administered as described in Figure 5. Shaded areas represent the mean values observed during the entire low-dose period and after 2 years of uninterrupted high-dose treatment (±1 SD). Normal range: 9–13 μmol/L.

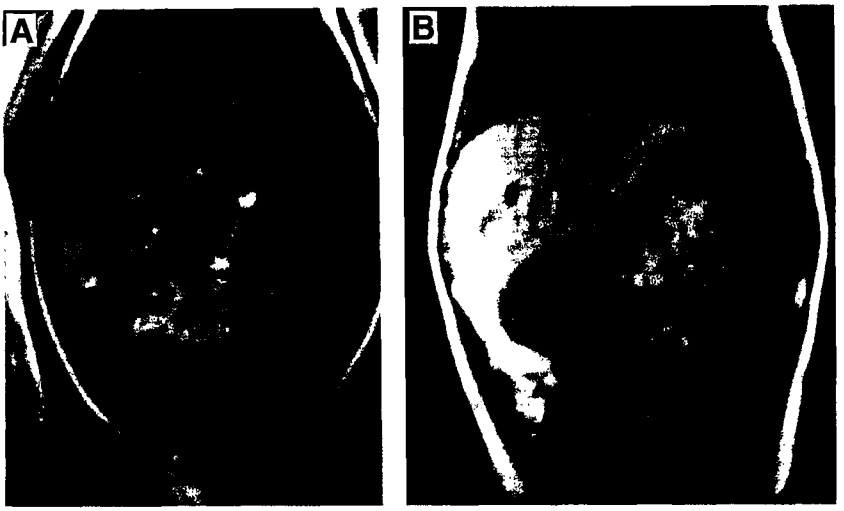

Figure 8 Comparison of T_1-weighted coronal magnetic resonance images of the abdomen obtained before and during high-dose enzyme supplementation (30 U/kg weekly). (A) Week 131; (B) week 316. A marked reduction in splenic size is evident.

first-order process; elimination half-lives ranged from 3.6 to 10.4 minutes. A typical infusion profile is illustrated in Figure 11. We observed that the steady-state concentration of enzyme in the plasma varied linearly as the infusion rate increased from 0 to 30 U/kg body weight. At the latter infusion rate, a steady-state enzyme concentration of 2×10^{-8} mol/L was observed, which closely approximates the K_d determined for the mannose receptor in equilibrium-binding studies in vitro. When the infusion rate exceeded 30 U/kg/h, the steady-state plasma concentration of enzyme rose exponentially, suggesting saturation of the clearance mechanism. Since saturation was not observed at infusion rates between 0 and 30 U/kg/h, these data imply that the amount of enzyme delivered to macrophages is directly proportional to administered dose within this range.

Significant reductions in hepatic glucocerebroside content occurred in 8 of 11 patients with intact spleens who underwent liver biopsy before and 44 hours after receiving enzyme doses between 30 and 234 U/kg body weight infused over 4 hours. In contrast, a reduction in lipid level was observed in only 1 of 10 patients who received doses between 0.6 and 24 U/kg. Specific activity measurements in liver biopsy specimens obtained 44 hours after infusion of macrophage-targeted glucocerebrosidase were compatible with a cellular elimination half-life

Figure 9 Effect of mannose-terminal glucocerebrosidase on the bone marrow. Right posterior iliac crest biopsy specimens were obtained before (Pre) and after (Post) 3-1/2 years of treatment with high-dose enzyme supplementation. Marked clearing of the Gaucher cell infiltrate is evident in the posttreatment specimen. Original magnification: ×8. (From Ref. 28.)

of approximately 8 hours for the exogenously delivered enzyme. For patients receiving infusions of 60 U of macrophage-targeted glucocerebrosidase per kg of body weight, a 100-fold increase in specific enzymatic activity could be estimated in liver 6 hours after completion of the infusion. Comparable measurements in transiliac bone marrow biopsy specimens revealed only a fivefold increase in specific enzymatic activity over basal values [30]. These data indicate that the tissue distribution of macrophage-targeted glucocerebrosidase is nonuniform and further support the view that clinical responses to enzyme replacement therapy in patients with Gaucher disease should be dose dependent and organ system specific.

VI. CLINICAL EFFICACY TRIAL

Since uniform reductions in hepatic glucocerebroside content were observed in the preceding trial at a dose of 60 U/kg body weight, we designed a chronic

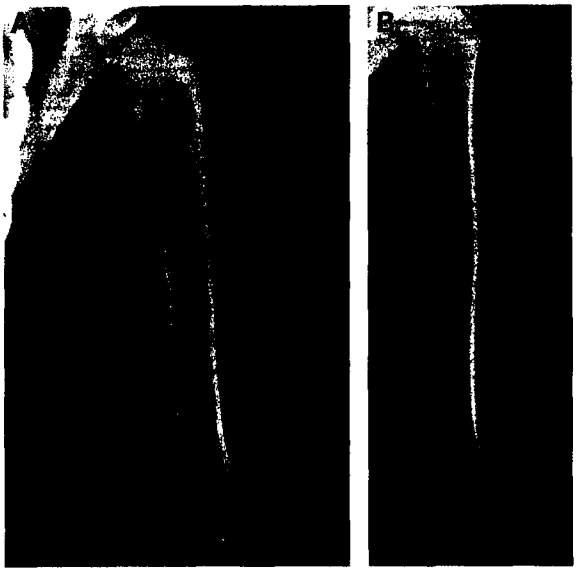

Figure 10 Comparison of skeletal involvement in a pretreatment radiograph of the left humerus (A) with an examination obtained on week 310 during high-dose enzyme supplementation (B). Radiographic signs of Gaucher disease are not apparent on the latter film. (From Ref. 27.)

infusion study to examine the long-term effects of administering this amount of macrophage-targeted glucocerebrosidase every other week to 12 patients with type 1 Gaucher disease. All of the patients participating in the trial had intact spleens. We chose several clinical and laboratory parameters characteristic of Gaucher disease to monitor the effects of the enzyme infusions. Measurements of each patient's height and weight, hemoglobin concentration, platelet count, serum acid phosphatase activity, and plasma glucocerebroside level were performed with each infusion. In order to quantify the visceral and skeletal responses to macrophage-targeted glucocerebrosidase, we expanded considerable effort in the development of accurate, noninvasive methods to measure changes in the volume of the liver and spleen [31], the extent of bone marrow infiltration by Gaucher cells [32,33], and the amount of cortical and trabecular bone [34]. The latter measurements were performed at 6-, 12-, 18-, or 36-month intervals. Four adults and eight children participated in the trial. Seven of the patients were female and five were male. One patient withdrew from the study for personal reasons after one year. After 9–12 months of treatment, the hemoglobin concentration

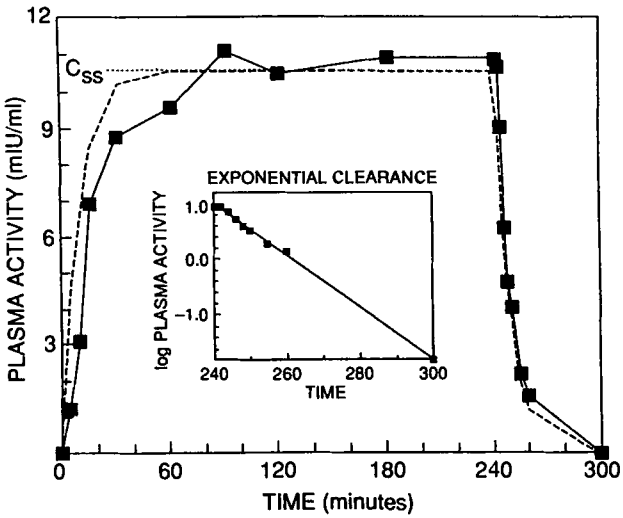

Figure 11 Enzymatic activity profile of mannose-terminal glucocerebrosidase in plasma of a boy with Gaucher disease. Enzyme was infused over 4 hours at a constant rate of 12.25 U/kg. A steady-state value of 10.6 mU/mL was achieved during the first hour. When the infusion was terminated at 240 minutes, enzyme was cleared from the plasma by a first-order process with an elimination half-life of 6.3 minutes. The dashed line represents the activity profile calculated from the observed kinetic constants.

increased significantly in all 12 patients and the platelet count increased in 7 [35]. Serum acid phosphatase activity decreased significantly in 10 patients during this period of time, and the plasma glucocerebroside level decreased in 9. Splenic volume decreased significantly in all patients after 6 months of treatment, and hepatic volume decreased in five. All patients noted an enhanced sense of well-being within the first 6 months of treatment [36], and the children gained substantial weight and grew taller.

Continuation of the enzyme infusions at a dose of 60 U/kg body weight every 2 weeks for an additional year produced a further reversal of the hematological and visceral abnormalities characteristic of Gaucher disease, although at a slower rate than during the first year of treatment (Tables 2 and 3). After 2 years of treatment at this dose, a *complete reversal* of anemia had occurred in 10 of 11 patients and the platelet count returned to fully normal values in 6. Plasma glucocerebroside levels decreased to normal values in all 11 patients, and serum acid phosphatase returned to normal in 3 patients. Splenic volume decreased by an average of 70% and hepatic volume by an average of 40% in comparison with

Table 2 Hematological Responses to Macrophage-Targeted Glucocerebrosidase: Time Course and Effects of Dosage Reduction

Time (months)	*N*	Hemoglobin (g/dL)	Platelets ($\times 10^3/mm^3$)	Acid Phosphatase (U/L)
0	12	8.9 ± 1.3	100 ± 69	5.7 ± 5.9
6	12	10.7 ± 1.1	112 ± 56	2.0 ± 1.1
12	12	11.9 ± 1.1	129 ± 59	1.5 ± 1.0
24	11	12.6 ± 1.4	161 ± 69	1.0 ± 0.7
33	11	12.8 ± 1.3	151 ± 59	1.1 ± 0.6
42	11	12.8 ± 1.4	139 ± 52	1.1 ± 0.6

Data are presented as mean values ±1 SD.
Enzyme dosage: 60 U/kg every 2 weeks: 0–24 months
30 U/kg every 2 weels: 24–33 months
15 U/kg every 2 weeks: 33–42 months
Normal ranges: Hemoglobin: 11.5–15.5 g/dL (age 7–14 y)
12.0–16.0 g/dL (age ≥ 15 y for women)
14.0–16.5 g/dL (age ≥ 15 y for men)
Platelets: $154–354 \times 10^3/mm^3$
Acid phosphatase: 0–0.4 U/L

basal values. As visceromegaly receded, the abdominal contour improved markedly, as illustrated in Figure 12.

After we had achieved a stable reversal of disease in these patients, the dose of enzyme was empirically reduced by 50% every 9 months in order to determine the minimum amount required for biochemical control of the illness [29]. All patients remained clinically and biochemically stable for 9 months at an enzyme dose of 30 U/kg every 2 weeks. However, 9 months after the dose was reduced to

Table 3 Visceral Responses to Macrophage-Targeted Glucocerebrosidase: Time Course and Effects of Dosage Reduction

Time (months)	*N*	Hepatic volume	Splenic volume
0	12	2.4 ± 0.4	49.7 ± 28.0
6	12	2.0 ± 0.4	28.9 ± 16.3
18	11	1.5 ± 0.3	15.3 ± 7.7
36	11	1.3 ± 0.2	11.2 ± 5.5
42	11	1.2 ± 0.2	9.5 ± 4.4

Data are presented as mean fold enlargement relative to normal values for body weight ±1 SD.
Enzyme dosage: 60 U/kg every 2 weeks: 0–24 months
30 U/kg every 2 weels: 24–33 months
15 U/kg every 2 weeks: 33–42 months

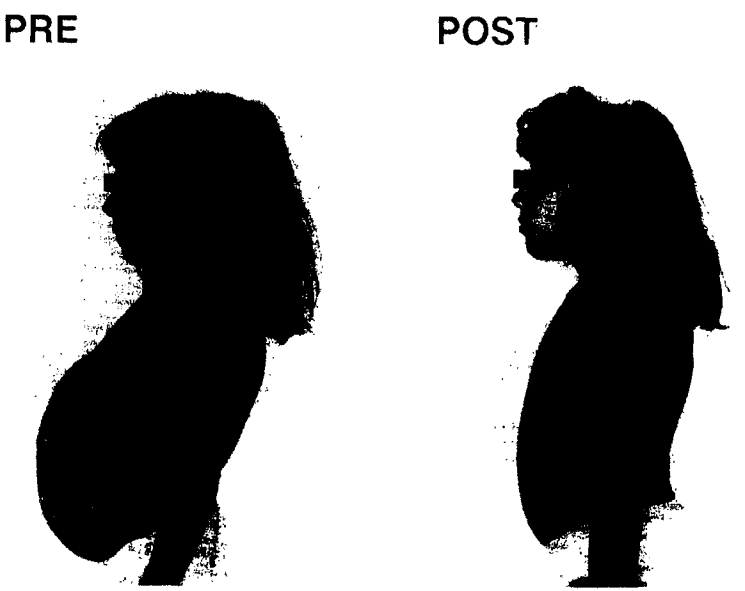

Figure 12 Photograph of an 8-year-old girl with Gaucher disease before (PRE) and after (POST) 26 months of treatment with macrophage-targeted glucocerebrosidase at a dose of 60 U/kg every 2 weeks. Marked reduction in visceromegaly is evident.

15 U/kg every 2 weeks, 3 of 11 patients developed recurrent signs and symptoms of the illness. These included a gradually decreasing platelet count, slowly increasing splenic volume and recurrent dull pain in the bones. Modest increases in enzyme dose to 20–30 U/kg every 2 weeks provided adequate long-term maintenance therapy in the latter patients.

As suggested by our initial investigations [27,28], reversal of skeletal pathology occurred slowly in the chronic infusion study when compared with the time course of the hematological and visceral responses to enzyme replacement therapy [30]. After 6 months of treatment at a dose of 60 U/kg every 2 weeks, biochemical improvement in the bone marrow was demonstrable in paired biopsy specimens obtained from two patients. Although a twofold increase in triglyceride and a fivefold decrease in glucocerebroside content were observed in these specimens, noninvasive imaging studies were not informative at this time. However, after 42 months of treatment, a dramatic normalization of the marrow fat content, assessed by quantitative chemical shift imaging, was observed in all 11 patients participating in the trial (Table 4). In addition to the marrow response, net increases in either cortical or trabecular bone mass, as assessed by

Table 4 Quantitative Skeletal Responses to Macrophage-Targeted Glucocerebrosidase

Time (months)	N	Marrow fat fraction lumbar spine[a] (%)	Cortical bone thickness mid-femur[b] (% normal)	Trabecular bone density lumbar spine[c] (% normal)
0	12	7.3 ± 6.7	64 ± 15	92 ± 13
42	11	22.9 ± 6.6	86 ± 18	98 ± 11

Data are presented as mean values ±1 SD. Enzyme was administered as specified in Table 3.
[a]The marrow fat fraction of the lumbar spine (L_2–L_5) was determined by quantitative chemical shift magnetic resonance imaging (QCSI). The normal range for healthy young adults is 23–35% [32].
[b]Combined cortical bone thickness at the level of the midfemoral diaphyses was measured on plain radiograms and expressed as a percentage of the average value for healthy age-matched controls [34].
[c]Trabecular bone density in the lumbar spine (L_1–L_4) was measured by dual energy quantitative computed tomography (DEQCT) and expressed as a percentage of the average value for healthy controls of the same stage of sexual maturity [30,34].

combined cortical thickness measurements and dual energy quantitative computed tomography, respectively, were observed in 10 patients. The latter responses are particularly important for children, since they suggest that a normal peak bone mass can be achieved by early adulthood if appropriate treatment is begun before fusion of the epiphyseal growth plates.

No untoward effects were encountered during the chronic infusion study [35,37]. Later, when a commercially available preparation of macrophage-targeted glucocerebrosidase (CEREDASE™) was administered to a much larger group of patients, 13% of them developed IgG antibodies to the enzyme [38]. Seroconversion usually occurred within the first year of treatment and did not appear to diminish the effectiveness of the enzyme. Approximately one quarter of the patients who developed antibodies in the latter study experienced periodic symptoms suggestive of immediate hypersensitivity during enzyme infusion. Symptoms typically included hives, flushing, itchiness, and chest and stomach discomfort. Administration of an antihistamine before the infusion and decreasing the rate of enzyme delivery usually eliminated these allergic phenomena.

VII. HOW BROAD IS THE CLINICALLY EFFECTIVE DOSAGE RANGE?

The salutary effects of enzyme replacement therapy for patients with type 1 Gaucher disease have been fully confirmed in numerous independent trials

[39–45]. Beneficial hematological and visceral responses have been observed over a broad range of enzyme doses (30–120 U/kg monthly) and frequencies of administration (three times weekly to every other week). Comparison of the hematological and visceral responses in patients receiving macrophage-targeted glucocerebrosidase in doses ranging from 30 to 60 U/kg every 2 weeks suggested that clinical outcome was independent of the amount of enzyme administered [39,40]. Likewise, frequent administration of low doses of enzyme (2.3 U/kg three times weekly) produced responses that superficially appeared comparable to those obtained by other investigators with doses of 60 U/kg every 2 weeks [41–43]. These observations have led to substantial controversy regarding the optimum dosage of macrophage-targeted glucocerebrosidase and its frequency of administration. Available data are insufficient to reach definitive conclusions regarding these issues. Several factors contribute to this dilemma, and it is instructive to review them since similar difficulties can be anticipated as therapies are developed for other metabolic storage disorders. Comparative studies of the clinical responses to macrophage-targeted glucocerebrosidase are limited by the striking biological variability of Gaucher disease, the heterogeneous nature of the responses observed among patients receiving any given dose of enzyme, and the lack of standardized methodologies to assess treatment outcome. Carefully designed prospective trials in which comparisons are made between more homogeneous patient groups using well-validated outcome measurements are necessary to overcome these difficulties. Application of the latter approach in a small study designed to assess the effect of frequency of enzyme administration on treatment outcome was enlightening. No difference in response was observed between patients receiving macrophage-targeted glucocerebrosidase at a dose of 2.3 U/kg three times weekly in comparison with 15 U/kg every 2 weeks [46]. These data suggest that highly frequent enzyme administration does not enhance its effectiveness. The total dose of enzyme administered over a given period of time, however, is important. In a comparative study of 10 patients with intact spleens who were treated with 10 U/kg every 2 weeks, we observed partial hematological and visceral responses in some, but not all, participants 6 months after initiation of therapy [47,48]. Strikingly more robust increases in hemoglobin concentration and reductions in organ volume were observed in patients treated with 60 U/kg every 2 weeks (Table 5). Demonstration of these highly significant differences in outcome was facilitated by selection of enzyme doses at the limits of the potential therapeutic range.

General principles of enzyme replacement therapy for type 1 Gaucher disease can be developed at this time from the data presented in the preceding sections. Because the disease and its response to treatment are heterogeneous, the management of each patient must be individualized. At the outset of treatment, enzyme dosage should be selected based on the overall burden of disease and the

Table 5 Effects of Enzyme Dosage on Hematological and Visceral Responses to Macrophage-Targeted Glucocerebrosidase

Enzyme dosage	N	Hemoglobin (g/dL)		Percent volume reduction	
		Baseline	6 Months	Liver	Spleen
10 U/kg	10	10.3 ± 0.4	11.3 ± 0.9	5.7 ± 3.7	11.7 ± 8.3
60 U/kg	10	9.2 ± 1.0	11.2 ± 0.9	15.0 ± 9.2	32.5 ± 11.4

Specified doses of macrophage-targeted glucocerebrosidase were administered every other week in two groups of patients with comparably severe type 1 Gaucher disease. Significant differences were observed in the magnitude of the hematological response ($p = 0.02$) and percentage reduction in hepatic ($p = 0.01$) and splenic ($p < 0.001$) volume after 6 months of treatment.
Source: Ref. 48.

desired clinical outcome. The typical therapeutic range is 10–60 U/kg every 2 weeks. Clinical responses appear to be dose dependent and organ system specific. Hematological and visceral improvement substantially precedes the skeletal response. Once objective reversal of disease has been achieved, the dosage of enzyme can be reduced in many patients to maintenance levels of 15 U/kg every 2 weeks. The dose of enzyme selected at the initiation of treatment and during the maintenance phases of therapy must be based on objective measurements of disease burden. A fixed dose of enzyme for all patients is inappropriate. As additional information is obtained from prospective trials, these therapeutic principals will no doubt be modified and further refined. At the moment, however, they provide practical guidance for the individualization of treatment.

VIII. ADDITIONAL PROJECTS AND FUTURE INVESTIGATIONS

Because of potential limitations in the supply of acceptable placentae and the remote possibility of infective contaminants, it was of great interest to determine whether macrophage-targeted glucocerebrosidase produced from recombinant sources would be as effective as the natural product. We therefore conducted a collaborative study with investigators at Mount Sinai School of Medicine in New York using glucocerebrosidase that had been produced recombinantly in Chinese hamster ovary cells. The enzyme obtained from these cells was modified by exposure to the same three exoglycosidases used previously to produce mannose-terminal placental glucocerebrosidase (Fig. 2). The clinical effectiveness of the recombinant macrophage-targeted enzyme was found to be

comparable to the natural product [49]. When the recombinant product becomes commercially available in large quantities, its use is anticipated to supplant the natural product.

The potential benefits of enzyme replacement therapy in neuronopathic forms of Gaucher disease have received limited study. Patients with acute neuronopathic, type 2 Gaucher disease appear to derive limited benefit from treatment [5,50]. We have found that patients with chronic neuronopathic, type 3 Gaucher disease show dramatic improvement in hematological and visceral abnormalities when treated with macrophage-targeted glucocerebrosidase. Long-term effects of enzyme replacement on the neurological signs in these patients, including horizontal gaze palsy, myoclonic seizures, and cognitive dysfunction, are currently being assessed. If the latter efforts are not successful, methods will have to be developed to more efficiently deliver enzyme to the central nervous system.

IX. CONCLUSIONS

Several important principles were established in the course of developing effective enzyme replacement therapy for Gaucher disease. The use of a cell-specific lectin to target intravenously injected glucocerebrosidase to sites of lipid storage in Gaucher disease provides a paradigm that will likely be used for the treatment of patients with other metabolic disorders. To assure the success of this approach, neoglycoforms of glucocerebrosidase had to be developed. Sensitive and reliable methodologies to assess the clinical and biochemical effects of enzyme replacement were also required. We anticipate that the lessons learned during these investigations will be applicable in the design of treatments for other heritable storage disorders.

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12
Deoxyribonuclease I

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I. INTRODUCTION

Deoxyribonucleases (DNases) catalyze the hydrolysis of phosphodiester linkages in deoxyribonucleic acids. Deoxyribonuclease I (DNase I), also called Dornase and classified as EC 3.1.21.1 (formerly EC 3.1.4.5), is one of the most thoroughly studied endonucleases. Within the DNA strain, it catalyzes the hydrolysis of phosphodiester linkages at the P-O3′ bond, producing 5′ oligonucleotides (Fig. 1). Like many other nucleases, DNase I requires divalent cations and shows optimal activity at neutral pH in the presence of Ca^{2+} and Mg^{2+} or Ca^{2+} and Mn^{2+} [1,2]. DNase I prefers double-stranded DNA, and by cutting only one strand at a time, single-strand nicks are produced. At later stages of the reaction, when the double-stranded substrate gradually disappears, the less susceptible single-stranded DNA is degraded by DNase I, but at a much slower rate (about 1000×). This phenomenon is thought to be responsible for the autoretardation of the enzyme [1].

Bovine pancreatic DNase I was purified in 1945 [3], and it was the first DNase to be crystallized [4]. Until the availability of recombinant human DNase (rhDNase) in the late 1980s [5], bovine pancreas remained the principal source of DNase I, for biochemical as well as for clinical investigations.

Experiments in the 1950s and 1960s showed that DNA is present in purulent lung secretions, while it is absent in noninfected secretions [6,7]. As early as 1950, Armstrong reported that bovine pancreatic DNase I significantly reduced the viscosity of purulent lung secretions in vitro [8]. These important

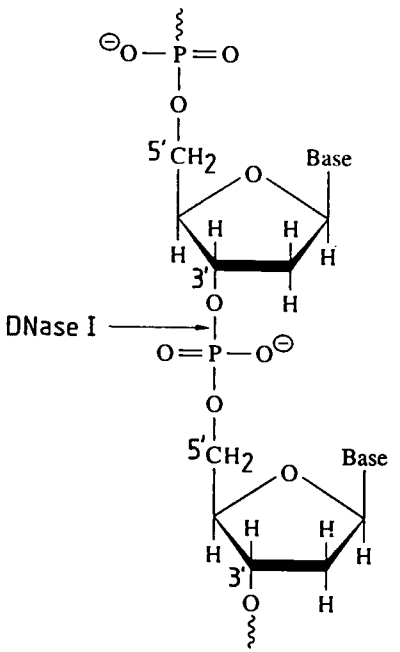

Figure 1 DNase I cleavage site. Hydrolysis by DNase I generates a 5′-phosphate and a 3′-hydroxyl terminus.

observations formed the basis for the renewed interest in therapeutic utility of this well-known enzyme.

In this chapter, we will focus on the present insights into the structure-activity relationship and the clinical situation of this enzyme without attempting to include all relevant studies. For reviews on the biochemistry up to 1980, we largely refer to the work of M. Laskowski [1], who was closely involved in the early studies on DNase I, and of S. Moore [9]. Moore shared with W. Stein the Nobel Prize in Chemistry in 1972 for their work on the chemical structures of pancreatic ribonuclease and deoxyribonuclease [10].

II. METHODS FOR DETERMINATION OF DNASE ACTIVITY

Various methods have been developed for the determination of DNase activity. A first group is based on the "hyperchromicity" method of Kunitz and determines

the increase in absorbance at 260 nm upon denaturation and depolymerization of DNA [4]. Other procedures measure the production of acid-soluble radioactivity from radiolabeled DNA by the enzyme or use viscosimetric, fluorometric, or colorimetric methods to quantify DNA hydrolysis [11–14]. Even a clot assay has been described [15]. The development of rhDNase as a therapeutic enzyme required the availability of a versatile assay method, useful over a wide activity range and suitable for high throughput of samples. A colorimetric method based on the decoloration of DNA complexed with methyl green [13] was modified for this purpose by Sinicropi [16].

III. PHYSICOCHEMICAL PROPERTIES

Early preparations of bovine pancreatic DNase I were shown to consist of several forms of DNase I. The principal component, a glycoprotein with a Mr of about 31,000, named DNase A, was accompanied by fractions B and C and very minor amounts of fraction D [17]. Specific activities were comparable. Fractions A, B, and C were separated and studied in more detail. They all have the same N- and C-terminal residues and one single N-linked carbohydrate. DNases A and B have the same amino acid composition but differ in carbohydrate structure. DNase B contains an extra sialic acid and galactose compared to DNase A. DNase C differs from DNase A only in a single amino acid substitution (His to Pro) [17]. The main component of bovine pancreatic DNase I, namely, DNase A, formed the starting material for further biochemical and structural investigations [2,18]. The complete amino acid sequence and the location of the disulfide bridges were determined by Liao et al. in the early 1970s [18]. The first disulfide bridge (Cys101-Cys104) is not essential for enzymatic activity, but the second one (Cys173-Cys209) is important for the enzyme structural integrity and activity [18,19]. Under controlled conditions, chymotrypsin can cleave the enzyme between Trp181 and Ser182 without inactivation. Both parts remain together and residues 177 to 181 can even be removed while maintaining a protein with more than 80% of its initial activity [20,21]. DNase I resembles RNase in that an intact C terminus is essential for correct folding of the protein.

The advances in the field of molecular biology allowed cloning of the gene for human DNase from a pancreatic cDNA library in the late 1980s [5]. A full-length clone of 1039 base pairs was isolated, encoding for a polypeptide of 260 amino acids with a high homology (77%) to bovine DNase I (Fig. 2).

rhDNase was expressed in human kidney embryonic 293 cells and also in Chinese hamster ovary (CHO) cells. Today the latter system is used for manufacturing the rhDNase I for therapeutic use (Pulmozyme®) [22].

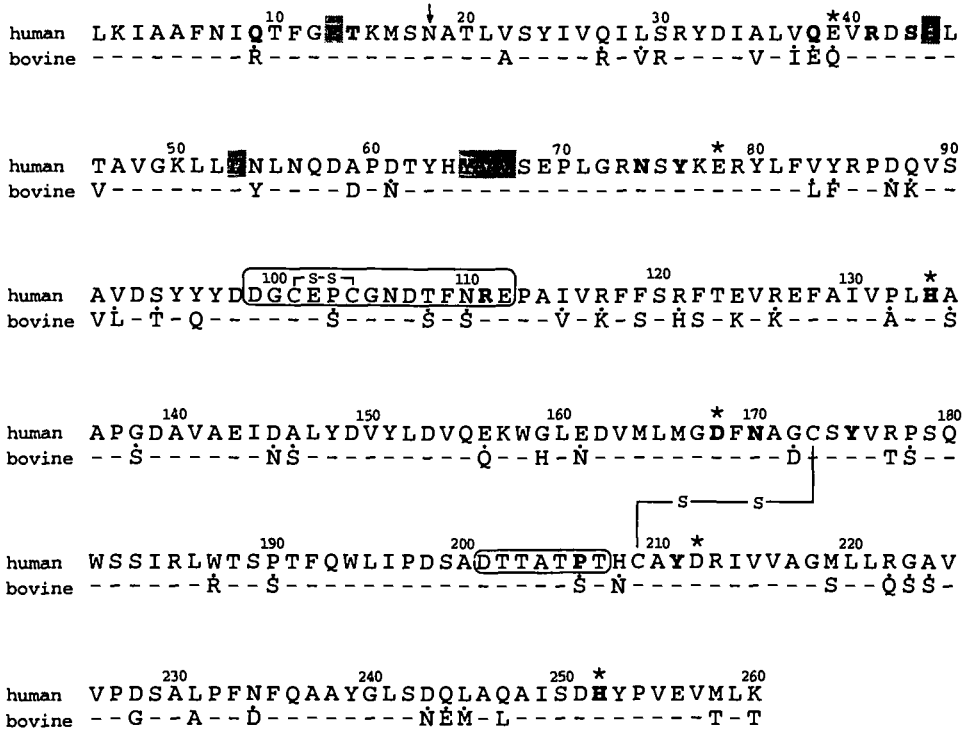

Figure 2 Primary sequences of human and bovine DNase I. Identical residues are not mentioned for the bovine sequence and are indicated (–); homologous residues have a point (·) above their symbol in the bovine sequence. The arrow at Asn18 shows the glycosylation site. Residues marked with an asterisk (*) are involved in the catalysis. Amino acids in bold take part in the DNA binding, while the shaded residues participate in the interaction with actin. The Ca^{2+} binding regions are outlined.

The three-dimensional structure of bovine pancreatic DNase I was first determined at 2.5 Å resolution by x-ray diffraction from single crystals [23]. DNase I proved to be a compact α,β-protein with two six-stranded β sheets packed against each other forming the core of a "sandwich"-type structure. The two mainly antiparallel β sheets are flanked by three longer and five short α-helices and extensive loop regions (Fig. 3).

Under crystallization conditions, two Ca^{2+} ions were bound to the enzyme [23]. This was expected after the observation of Price, who reported that DNase I has two strong Ca^{2+} binding sites at pH 7.5 besides several weaker sites [24]. One

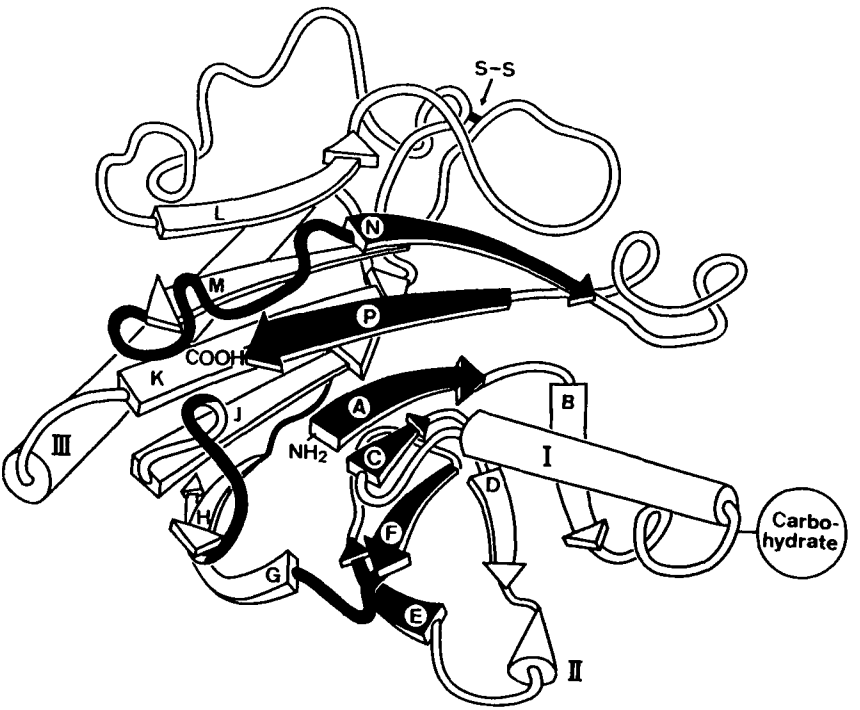

Figure 3 Three-dimensional structure of DNase I. β-Strands (marked by capital letters) are represented by arrows; helices are represented by cylinders. Two six-stranded β-pleated sheets consisting of strands E, F, C, A, P, N (sheet 1) and strands G, H, J, K, M, L (sheet 2) are packed against each other, forming the core of the enzyme. The flexible loop region connects β-strands H and G. The carbohydrate side chain is attached to Asn18 at the beginning of helix I. The disulfide bridge between Cys173 and Cys209 is indicated. (From Ref. 23.)

of the sites is located near a flexible loop and seems to coordinate with Asp99, Glu112 and to the side chain of Asn105. The second Ca^{2+} binding site is located close to the essential disulfide binding between Cys173 and Cys209, in the loop formed by residues 201–209. Apart from ligation to Asp201, there seems to be an interaction with Thr203 and Thr207. These Ca^{2+} ions bound to DNase I under crystallization conditions are important for its structural integrity by stabilizing the surface loop Asp201 to Thr207 and limiting the region of high thermal mobility in the flexible loop to residues Gly100 to Gly105 [23–26]. These Ca^{2+} binding sites are shown in Figure 2. In the neighborhood of His134, which was

shown to be essential for the catalytic activity [27,28], another cation-binding site was found. In the three-dimensional structure, two carboxylate side chains (Asp42 and Asp251) are located in the vicinity, providing ligands for the cations. The binding of Ca^{2+} to this site is very week in the absence of nucleotide. This observation, together with the vicinity of the "active" His134, led to the hypothesis that this Ca^{2+} binding site is involved in the catalytic mechanism of the enzyme [26].

Derivatization experiments revealed that the εNH_2 groups are not essential per se. However, removal of positive charges on the enzyme progressively inactivates the DNase I [29]. Deamidation of the Asn residues occurs during the production of rhDNase I, primarily during the cell culture process and subsequent purification and storage. The fully deamidated Pulmozyme® is reported to be half as potent as the nondeamidated form. The commercial Pulmozyme® is 65 ± 17% deamidated [22].

Most of the differences between the human and bovine pancreatic DNase I are located at the surface of the molecule in hydrophilic regions, which might be immunogenic [5]. In the past, the development of anti-DNase I antibodies was reported in patients who received multiple doses of bovine DNase I [30]. This immunogenicity, together with adverse reactions against impurities (often proteases) in the preparations of bovine DNase I, prohibited a thorough clinical investigation of this enzyme (Sec. V) [31,32].

IV. DNASE I INTERACTIONS

A. DNA–DNase I Interaction–Proposed Catalytic Mechanism

Bovine DNase I, which shows a preference for double-stranded DNA, cleaves this substrate with strongly varying cutting rates [33]. Hence, the enzyme is thought to recognize sequence-dependent structural variations of the helix geometry. Cleavage probabilities are very low in regions of DNA consisting of runs of consecutive adenosines or thymidines and are also lowered in guanosine, cytidine-rich regions. Efficiency is highest in mixed-sequence regions. X-ray crystal data showed that DNase I interacts with the DNA helix by binding in the minor groove, which is filled by residues Arg41 and Tyr76 from DNase I (Fig. 4) [26]. The width and depth of the minor groove may affect the hydrolysis rate by DNase I. Mutagenesis studies suggest that helical stiffness or bendability is also involved [34,35]. Suck studied the three-dimensional structures of two DNase I–oligonucleotide complexes [34,36] and postulated in his review paper that only a combination of local and global helix parameters will fully account for the

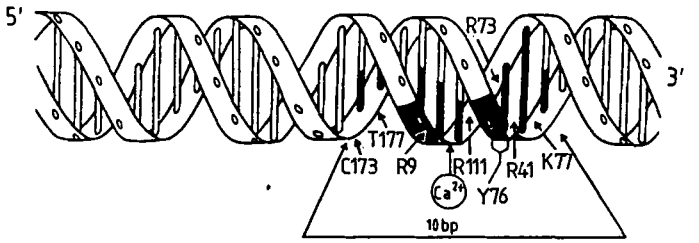

Figure 4 Schematic representation of DNase I–DNA interactions. Approximately 10 base pairs (bp) of the DNA are in contact with DNase I on one side of the double helix (indicated in black). Side chains of DNase I residues interacting with the DNA are indicated. (Adapted from Ref. 26.)

difference in cutting rates [37]. Upon binding of the oligonucleotide, no significant conformational change takes place in the DNase I, while on the other hand, considerable distortions are induced in the DNA. Being an intrinsically flexible substrate, DNA adapts to the rather rigid enzyme. The exposed loop around Tyr76 of DNase I binds tightly in the minor groove of a B-type DNA. The stacking-type interaction between Tyr76 and a deoxyribose ring is the main reason for the distortion. Only one side of the double helix makes direct contact, and this happens over a length of six base pairs. There are no direct contacts with the major groove, but a few base pairs further, both approach each other again. Upon binding to DNase I, the DNA is bent away from the enzyme toward the major groove, thus widening the minor groove. Residues of DNase I known to be involved in DNA binding and/or catalysis are shown in bold in Figure 2 [29,34,36,38].

The width of the minor groove and the helical stiffness cannot explain the rather drastic differences in cutting rates at the neighboring phosphodiester bond. Locally, the orientation of the scissile phosphate group seems important for cleavage rates. The cleavage of the P-O_3' bond by DNase I proceeds via an inversion of configuration at the phosphorous group. This suggests the reaction involves an in-line nucleophilic attack by a water molecule opposite O_3'. Weston et al. proposed a general acid-base catalysis as outlined in Figure 5 [36]. One histidine (His252) acts as a general base, abstracting a proton from the attacking water, and a second histidine (His134) functions as a general acid and protonates the leaving O_3'. The metal ion bound in the active site region can stabilize the transition state. Residues Arg9, Glu39, and Asp251 may also participate in this stabilization process.

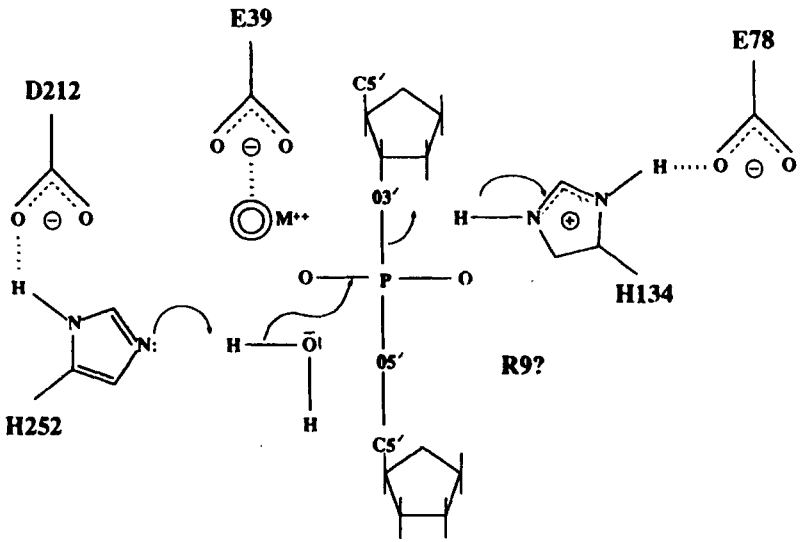

Figure 5 Scheme of a possible mechanism for the catalysis by DNase I. From the disposition of the residues and assuming that the reaction involves an in-line attack at the phosphorus group by a water molecule, it seems most likely that His252 acts as a general base to abstract a proton from a water molecule opposite the O3′ atom. The subsequent attack at the phosphorus group with inversion of its configuration gives rise to a penta-covalent transition state that may be stabilized by the metal ion and possibly by the side chain of Arg9. His134 is in an ideal position to protonate the leaving O3′. The metal ion (double circle denoted M^{++}) may also be important for the correct positioning of the phosphate group. (From Ref. 36.)

B. Actin–DNase I Interaction

Years ago, a naturally occurring protein was described that inhibited DNase I activity [1,9]. Lanzarides and Lindberg identified the molecule as cytoplasmic actin [39]. This interaction proved to be specific as no other DNA-degrading endonuclease has been found to be inhibited by actin. DNase I can depolymerize filamentous actin to form a stable complex consisting of 1 mol DNase and 1 mol globular actin [40]. The binding constant between fluorescently labeled DNase and monomeric actin (rabbit muscle) was estimated to be around 5×10^8 M^{-1}, and the inhibition is competitive [41]. This very stable complex can be separated by 5′ nucleotidase, resulting in a reactivation of DNase activity [9]. Kabsch et al. reported on the atomic structure of the actin–DNase I complex [42]. Actin (Mr 49,000) consists of two domains of almost the same size. The major contacts

between both molecules consist of hydrogen bonds and electrostatic and hydrophobic interactions. The residues of DNase I known to be involved in the binding are indicated in Figure 2. A major binding occurs between Tyr65, Val66, and Val67 of DNase I and residues Gly42, Val43, and Met44 of actin, which are incorporated as a parallel strand of α/β sheet [42,43]. Atoms involved in electrostatic interactions and hydrogen bonds are shown in Figure 6. The N and C termini of actin are in spacial proximity to each other, but far away from the binding region to DNase I. Structural analysis of the actin–DNase I complex can explain a lot of biochemical findings, including the inhibition of DNase activity upon actin binding. Ulmer and colleagues constructed a model of a temary complex between DNase I, DNA, and actin. It shows that the DNA and actin-binding sites are adjacent but distinct [44]. These authors engineered human DNase I to create two classes of mutants: one that was resistant to inhibition by globular actin and another class of active site mutants. The relative specific activities of the variants was measured in comparison to wild-type rhDNase I. The actin-binding site variants had similar specific activity to the wild-type enzyme, and the active site variants bound with the same high affinity to G-actin as the normal rhDNase I. The different rhDNase variants were compared for their efficiency to reduce the viscoelasticity of cystic fibrosis sputum in vitro (Fig. 7). The actin-resistant variants were more potent (10–50 times) in reducing the viscoelasticity of sputum. This observation clearly shows that G-actin is a significant inhibitor of DNase I in sputum [44]. The value of engineering rhDNase to optimize its clinical efficiency remains to be explained.

V. PHYSIOLOGICAL ACTION OF ADMINISTERED DNASE I

A. Clinical Use of DNase I

1. DNase I for Treatment of Respiratory Diseases

a. The influence of DNase I on cystic fibrosis sputum

A major respiratory disease for which treatment with DNase I can be considered is cystic fibrosis (CF) [45,46]. The CF defective gene leads to a dysfunction of the exocrine secretory glands, often resulting in both severe gastrointestinal as well as respiratory clinical symptoms. Patients suffer from the obstruction of the airways with a dehydrated and viscous mucus, often facilitating the persistence of severe infections of the lung. The introduction of antibiotics substantially improved patients' prognosis, but antibiotherapy has not been able to prevent the airways from becoming permanently colonized by typical bacterial flora. Due to such infections, massive numbers of leukocytes are recruited to the respiratory

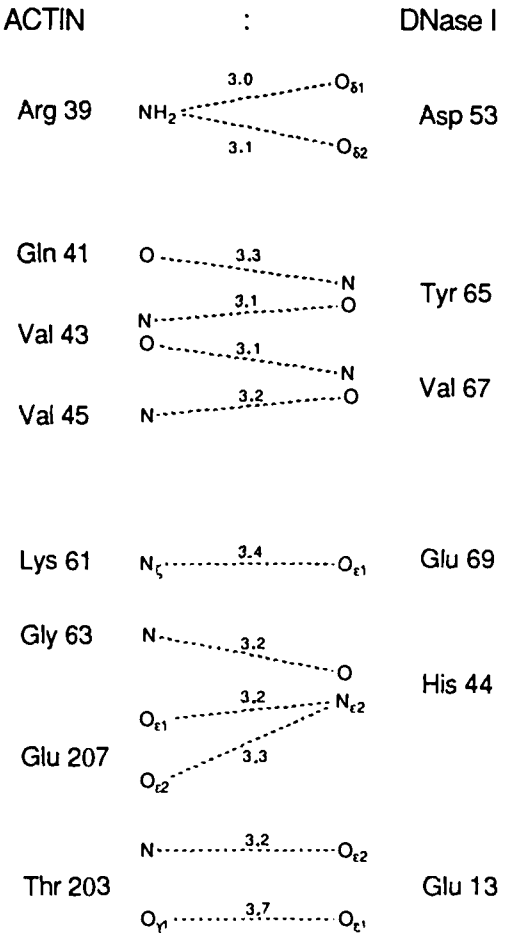

Figure 6 Binding regions between actin and DNase I. Atoms involved in hydrogen bonds and electrostatic interactions between the two proteins. Distances are given in Å. The actin residues Gly42, Val43, and Met44 are incorporated as a parallel strand of a β-pleated sheet in DNase I with partners Tyr65, Val66, and Val67. (From Ref. 42.)

tract. The degeneration of the leukocytes releases large amounts of DNA, which greatly exacerbates the mucus viscosity [6,7,50,51]. Experiments in the 1950s and 1960s showed that DNA is present in purulent lung secretions but absent in noninfected secretions [6,7]. Armstrong described in 1950 how bovine pancreatic DNase I (contaminated with proteases) significantly reduced the viscosity of purulent lung secretions in vitro [8]. These findings were confirmed by Chernick

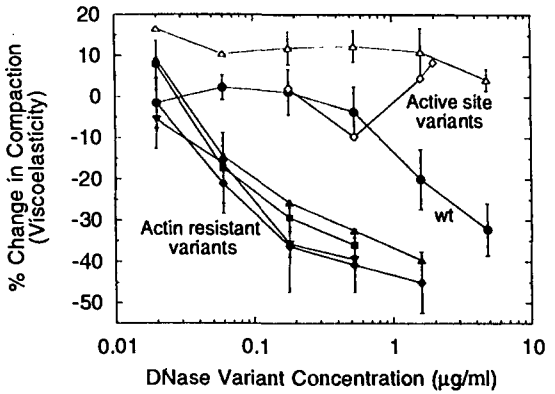

Figure 7 Effects of variants of rhDNase I on the compaction of sputum from cystic fibrosis patients. The percent compaction of the sputum (mean ± SEM), which correlates with sputum viscoelasticity, is plotted versus the variant concentration; wild type (•); active site variants: His134Ala (Δ) and His252Ala (◆); and actin-resistant variants: Asp53Arg (■), Tyr65Ala (◆), Tyr65Arg (▲), and Val67Lys (▼). (From Ref. 44.)

et al. [52]. Based upon their experiments and upon clinical studies in patients [53], bovine pancreatic DNase I was approved for human use in the United States in 1958.

Generally, inhaled bovine pancreatic DNase I was well tolerated. However, after a while adverse reactions were reported both after inhalation [54–56] as well as after intrathecal administration [31]. Trials were stopped because of these adverse effects, which may have been related (1) to the fact that the final product contained, besides the bovine pancreatic DNase I, up to 2% trypsin and chymotrypsin [31,32] and (b) to the immunogenicity of the bovine DNase I. The role of contaminating proteinases in the reduction of the viscosity of purulent lung secretions remained unclear. Shak et al. clearly illustrated in vitro that both purified rhDNase I as well as bovine pancreatic DNase I, free of proteolytic activity, dramatically decreased the viscosity of CF sputum, which is transformed from a gel to a flowing liquid [5]. Even the addition of trypsin or chymotrypsin to rhDNase did not increase its ability to reduce the sputum viscosity. Several authors [57–59] suggested that the depolymerization of DNA by the addition of DNase I not only reduces the viscosity but also the elasticity and the adhesiveness of sputum collected from CF patients. This might be important, as transport of mucus by coughing depends not only on its viscoelasticity but also on its adhesive surface properties [60]. Shah et al. reported that rhDNase also reduces the sputum viscosity in CF patients in vivo after 10–14 days of inhalation

treatment [61]. In the sputum of patients treated with rhDNase, a large decrease in the amount of high molecular weight DNA has been observed [62]. Moreover, the in vivo effects of rhDNase clearly correlate with changes in pulmonary function [63].

b. Clinical results of DNase I therapy in cystic fibrosis

The clinical availability of rhDNase in the early 1990s gave rise to numerous clinical experiments. Since then, issues regarding the safety and efficacy of the enzyme have been addressed without the difficulties associated with the use of impure preparations from a nonhuman source. The first results of clinical studies with rhDNase I were reported in 1992 [64]. Bryson et al. [45], Hodson and Shah [65], Cramer et al. [66], Zach [67], and Gonda [68] published reviews on the therapeutic potential of rhDNase in CF. Generally, rhDNase is well tolerated, although some adverse effects are still present and are mainly involved with the respiratory tract. Voice alteration after long-term therapy remains the main side effect of rhDNase and leads to drug discontinuation in some cases [45,69].

After short-term therapy (some weeks), in CF patients with mild to moderate stable pulmonary disease, inhalation of rhDNase in combination with other CF therapies (e.g., bronchodilators, pancreatic enzymes, chest physiotherapy) improves the lung function [64,70–72]. The rhDNase therapy is also associated with significant improvements in general well-being, cough frequency, and congestion [71,72].

After long-term inhalation therapy, in CF patients with mild to moderate stable pulmonary disease, improvements in lung function observed within the first month of treatment stabilized over the next 5 months [73,74]. Whether long-term therapy significantly improves the well-being of these patients has not yet been firmly established from the available literature [67,73,75].

According to clinical studies with rhDNase on CF patients with severe pulmonary disease, the improvement in lung function did not differ significantly from the placebo effect after 2.5 mg daily administration for 14 days [69,76]. In 1994, Bryson et al. pointed out the need for longer-term studies in order to establish the efficacy of rhDNase treatment in CF patients with severe pulmonary diseases [45]. It has been reported that rhDNase therapy is well tolerated in CF patients with acute exacerbations requiring urgent hospitalization [77,78]. However, their study did not demonstrate a statistically significant therapeutic effect of rhDNase when added to a regimen of antibiotics and chest physical therapy, which agrees with earlier findings [45,76].

c. DNase I for treatment of non-CF respiratory diseases

Besides the use of bovine pancreatic DNase I and rhDNase for the treatment of CF, the enzyme was also investigated for the treatment of other respiratory

diseases. Bryson et al. reported that rhDNase was investigated in phase III studies in patients with bronchiectasis and acute exacerbations of chronic bronchitis [45]. Recently, Touleimat et al. [79] and Greally [80] reported cases in which rhDNase was successfully utilized for the management of lobar atelectasis by bronchoscopic instillation.

For inhalation treatment of respiratory diseases, a pharmaceutical DNase I aerosol is on the market. Pulmozyme® is a sterile solution for respiratory use at a concentration of 1000 Genentech Units/mL [22]. It contains 1 mg/mL rhDNase, sodium chloride as a tonicity modifier, calcium chloride as a stabilizer, and water for injection. Since deamidation is rapid at high pH and aggregation occurs at low pH, a nearly neutral solution (pH 6.3) is required. It is administered by means of a compressed air-driven nebulizer. Each 2.5-mL single-unit ampule will deliver 2.5 mg of rhDNase to the nebulizer chamber. The efficacy of DNase inhalation therapy largely depends on the aerosol quality and characteristics, which determine the respirable fraction. Significant differences were found between the different aerosol drug-delivery systems [68,81].

2. DNase I for Treatment of Nonrespiratory Diseases

Since 1969, bovine pancreatic DNase I has been used for the treatment of ulcers in dermatology [82–84]. It degrades the polynucleotides that are a main constituent of dead cells. One dermatological product based on DNase I is Elase®. This ointment contains both bovine pancreatic DNase I as well as a fibrinolytic complex formulated in a mixture of polyethylene glycol and paraffin oil [85].

In a recent publication Linardou et al. studied the role of recombinant DNase I in cancer therapy [86]. They showed that recombinant DNase I is an attractive candidate for tumor targeting since it is normally nontoxic but highly cytotoxic when redirected to the cell nucleus.

B. Pharmacokinetic Properties of Administered DNase I

As discussed above, the main therapeutic value of DNase I is in the treatment of respiratory diseases. As a consequence, most of the pharmacokinetic data available are on the pharmacokinetics of DNase I after inhalation. Pharmacokinetic studies on animals have been reviewed by Green [87]. Inhalation experiments with rhDNase I revealed a low bioavailability of <15% in rats and <2% in monkeys. Studies in rats have indicated that the half-life of rhDNase from the lung is about 11 hours. Based upon the results from a phase I clinical trial, Aitken et al. reported the DNase I concentration in serum only increased slightly after 5 days inhalation of 6–30 mg rhDNase per day [64]. The serum DNase levels did not differ significantly from the endogenous serum DNase levels as measured by Sinicropi et al. [88]. This suggested a low systemic exposure of DNase I in

humans after rhDNase inhalation and agreed with phenomena observed in animals. Assuming the absence of any specific mediated transport system, the low bioavailability after inhalation is not surprising considering the large molecular weight and the hydrophilic properties of this protein. Absorption of rhDNase from the gastrointestinal tract following oral administration to rats seemed negligible. From pharmacokinetic studies on rats, Mohler et al. suggested that rhDNase might be protein-bound in serum [89]. Studies in rats and monkeys illustrated that after intravenous administration, rhDNase was rapidly cleared from the serum [87].

VI. PROSPECTS

Since the discovery of the defective gene responsible for CF in the late 1980s [90–92], gene therapy has been proposed as a new strategy to correct the underlying defect in CF, which is currently the main therapeutic field for DNase I. Early clinical gene therapy work was recently discussed by Wagner et al. [93]. At the time being, it is unknown if gene therapy will be able to replace DNase I therapy for CF treatment. As Accurso stated [94], "Researchers are hopeful that gene therapy may eventually eradicate the bronchopulmonary component of CF. In the meantime, DNase I provides the clinician with an important therapeutic adjunct for CF patients with mild lung disease." On the other hand, a further tailoring of the promising actin-resistant variants of rhDNase may be of more early benefit to the CF patient. However, this hopeful approach needs additional biochemical exploration and awaits clinical investigation using carefully designed protocols. It is to be expected that the benefits of this important new adjunct in CF will be valuable in other pulmonary clinical conditions, such as mucus plugging in status asthmaticus.

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13

Therapeutic Protein Inhibitors of Elastase

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I. INTRODUCTION

Human neutrophil elastase (NE) is a powerful proteolytic enzyme capable of degrading a broad range of substrates including structural and matrix proteins [1–3]. Human neutrophils contain a number of proteolytic and oxidative enzymes, stored in granules, which aid in the defense mechanism during an inflammatory response. Elastase, cathepsin G, and the recently identified proteinase 3 are the main proteinases located within the azurophil granules of neutrophils. The primary role of NE appears to be the hydrolysis of protein substrates taken up by neutrophils during the process of phagocytosis. This function is deduced from the location of elastase within azurophil granules and due to its ability to digest bacterial proteins [4]. NE, however, has a broad substrate specificity and readily degrades elastin, collagen, fibronectin, laminin, and proteoglycans [3]. Thus, elastase may also have an extracellular function and play a role in neutrophil migration, connective tissue remodeling, wound debridement, and possibly complement activation.

Due to its broad specificity and high concentrations within neutrophils. NE has been implicated in the development and progression of several lung diseases such as pulmonary emphysema, cystic fibrosis, adult respiratory distress syndrome, and chronic bronchitis [2–4]. During phagocytosis and neutrophil turnover, there is a release of elastase into the extracellular environment which, if not controlled, can result in extensive damage to connective tissue. The destructive effect of elastase is normally controlled by endogenous proteinase inhibitors

such as α_1-proteinase inhibitor (α_1-PI) [5,6]. Under inflammatory stress, this proteinase-antiproteinase balance can shift in favor of free proteolytic activity resulting in abnormal degradation of extracellular matrix proteins. This imbalance can result either from an increase in the concentration of elastase or from reduced levels of endogenous inhibitor.

The therapeutic need for potent inhibitors of elastase has been recognized by several pharmaceutical companies. Administration of proteinase inhibitors to augment the normal endogenous levels should shift the balance in the direction of the inhibitor. One approach is the replacement or supplementation with natural or recombinant proteinaceous elastase inhibitors [7,8]. A second approach is the design and development of low molecular weight synthetic elastase inhibitors [9,10]. Both of these strategies are currently being pursued, with various inhibitors being evaluated in clinical trials. As the title suggests, in this chapter we will focus on the naturally occurring polypeptide inhibitors of elastase. The four inhibitors that will be described and reviewed as candidates for therapeutic agents are α_1-PI, secretory leukoprotease inhibitor (SLPI), elafin, and eglin c.

II. ELASTASE AND PULMONARY DISEASES

Human neutrophil elastase is the proteinase that has received by far the most attention over the last 30 years primarily due to the fact that it was thought to be the major factor involved in the development of emphysema. This assumption was derived from the observation of Eriksson in 1963 that individuals with an inherited deficiency of α_1-PI developed emphysema at an early age [11]. One year later, Gross showed that emphysema could be produced in experimental animals after intratracheal administration of papain, a plant proteinase with a broad substrate specificity [12]. These two observations led to the proposal of the proteinase-antiproteinase hypothesis [13], which assumes that there is a balance between elastase activity and the presence of controlling proteinase inhibitors. If this balance is shifted in favor of the elastinolytic activity, there is an uncontrolled degradation of elastin and other structural proteins, eventually resulting in emphysema. However, this elastase-antielastase hypothesis is overly simplified, and we now know that there are many other factors involved in lung tissue degradation. Nonetheless, the overall therapeutic approach is still to swing the balance toward inhibiting elastinolytic activity. There are numerous other diseases in which the proteinase-antiproteinase imbalance is believed to play a major role in pathogenesis. These include cystic fibrosis, adult respiratory distress syndrome, chronic bronchitis, asthma, and rheumatoid arthritis. The following section presents a closer look at some of the many factors involved in the development of emphysema and cystic fibrosis. Many of the processes that have

been described for emphysema and cystic fibrosis may also apply to other diseases, however, due to the spacial limitations of this chapter, they will not be discussed here.

A. Emphysema

Emphysema is characterized as an irreversible condition of the lung marked by progressive enlargement of the alveoli distal to the terminal bronchioles and destruction of the alveolar walls [14]. There are two main types of emphysema: familial (or hereditary) and acquired. Familial emphysema is caused by a homozygous genetic mutation in the α_1-PI gene leading to a deficiency of the inhibitor. These α_1-PI–deficient individuals have less than one-third the normal serum level of α_1-PI. Affected individuals consequently have insufficient amounts of α_1-PI to protect the lung tissue from degradation and are prone to develop emphysema in the third or fourth decade of life, even earlier if the individual is a cigarette smoker. The current treatment for patients with α_1-PI deficiency is infusion of partially purified human plasma α_1-PI at weekly or monthly intervals. This therapy has been shown to be an effective means to maintain serum α_1-PI levels within the normal range but above the critical inhibitory capacity believed to provide lung protection from NE [15].

Acquired emphysema is the most common type of emphysema, typically presenting with a centriacinar pattern of destruction affecting the upper half of the lungs and usually resulting from cigarette smoking. Although tobacco smokers have normal α_1-PI levels, which should protect against elastin degradation, 10–20% will nonetheless develop emphysema. Cigarette smoke contains irritants that cause a recruitment of neutrophils and macrophages to the lung, resulting in an increased proteinase burden. α_1-PI provides the main antiproteinase screen in the lower respiratory tract, while SLPI protects the upper airways. Both of these inhibitors have an active-site methionyl residue, which if oxidized to methionine sulfoxide dramatically reduces their inhibitory capacity. Neutrophils are capable of generating oxygen free radicals, and cigarette smoke itself contains oxidants that can inactivate the proteinase inhibitors and promote unrestricted proteinase degradation. Thus, cigarette smoke not only increases the number of neutrophils or level of elastase present in the lung but also decreases the protective effects of α_1-PI and SLPI by oxidative inactivation of these inhibitors.

There are several other factors that may play a role in the development of emphysema in α_1-PI–sufficient individuals, however, one can conclude that the proteinase-antiproteinase imbalance is involved, as in the case of hereditary emphysema. If the protective shield of the lung is diminished either due to oxidative or proteolytic inactivation of inhibitors, then uncontrolled elastinolytic activity will occur resulting in tissue damage (Fig. 1). This protective shield can

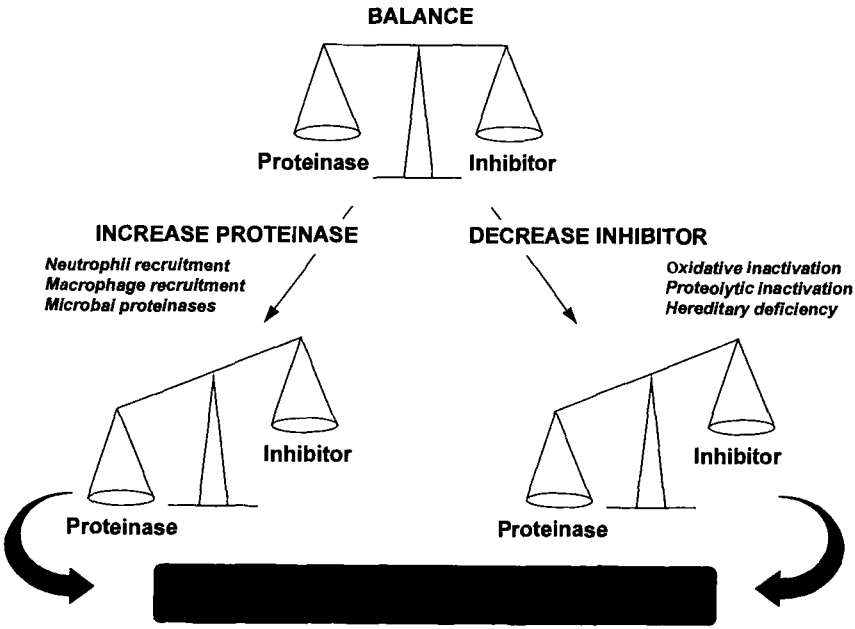

Figure 1 Proteinase-proteinase inhibitor balance. Normal protein degradation is controlled by endogenous proteinase inhibitors. The inhibitor capacity can be reduced by oxidative or proteolytic inactivation. These proteolytic inactivators can also degrade structural proteins. Chemoattractants are produced at the site of degradation, leading to macrophage and neutrophil recruitment.

be reestablished with the supplementation of proteinase inhibitors in order to swing the balance back toward the inhibitor. However, in the case of acquired emphysema, the first step to stop the progression of the disease is to refrain from smoking.

B. Cystic Fibrosis

Cystic fibrosis is an autosomal recessive disease caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene producing a dysfunctional chloride channel, normally located on the apical membrane of exocrine glands [16]. The disease manifests itself at an early age in the lung with accumulation of thick, sticky mucus and bacterial infections. These infections are followed by an inflammatory response, resulting in an infiltration of neutrophils

into the lower respiratory tract eventually leading to airway obstruction, pulmonary failure, and death. During this inflammatory response, activated neutrophils release large amounts of NE into the extracellular matrix leading to progressive tissue damage. Cystic fibrosis patients have normal levels of plasma proteinase inhibitors such as α_1-PI, however, it appears that these antiproteinase defenses are simply overwhelmed by the sheer load of neutrophils and bacterial elastases. Though the exact mechanisms are unclear, it is now recognized that the loss of pulmonary function in CF is due to the destruction of extracellular matrix proteins resulting from an accumulation of neutrophils and hence NE on the epithelial surface of the lung. The two pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa* frequently colonize the lungs of cystic fibrosis patients and secrete powerful proteinases, which rapidly inactivate both α_1-PI and SLPI by cleavage at or near the active center of the inhibitor [17]. These proteinases also possess elastinolytic activities, which may contribute to progressive pulmonary impairment.

Treatment of cystic fibrosis normally involves the use of antibiotics to reduce the bacterial load of the lung. In addition to new more potent antibiotics, a host of other strategies are now being developed, including aerosolized recombinant DNase and aerosolized proteinase inhibitors. Aerosolized installation of α_1-PI raises the inhibitor concentration to a level that completely eliminates free NE activity and increases the phagocytic ability of the neutrophil. These inhibitor studies suggest that instillation of proteinase inhibitors can be an effective means to reduce the NE burden, thus reducing the damage to the respiratory epithelial surface.

III. NEUTROPHIL AND MACROPHAGE ELASTASE

Elastase is the name given to proteinases that possess the ability to hydrolyze mature cross-linked elastin [18]. Elastin is an insoluble structural protein responsible for the elastic properties of the lung, skin, and arteries and is quite resistant to most proteinases. Elastin is high in hydrophobic amino acid residues such as valine, alanine, glycine, and proline [19]. Insoluble elastin fibers contain cross-links usually between four lysine residues, which form a unique cyclic product, desmosine. The presence of soluble desmosine cross-links in plasma can be used as a measure of elastin breakdown. Of all the elastases in humans, neutrophil elastase has received the most attention over the years due to its broad substrate specificity and abundance within the cell. However, neutrophils and macrophages contain several proteinases (Table 1), which are capable of degrading elastin.

Table 1 Human Proteinases that Possess Elastinolytic Activity

Proteinase	Cell source	Catalytic class
Elastase	Neutrophil	Serine
Cathepsin G	Neutrophil	Serine
Proteinase 3	Neutrophil	Serine
Collagenase	Neutrophil	Metalloproteinase
Gelatinase	Neutrophil	Metalloproteinase
Metalloproteinase	Macrophage	Metalloproteinase
Cathepsin B, L, S	Macrophage	Cysteine

A. Neutrophil Elastase

Neutrophil elastase is a member of the serine proteinase family. A characteristic common to all mammalian serine proteinases is the presence of the His-57, Asp-102, and Ser-195 (chymotrypsin numbering) residues forming the charge-relay system. This catalytic triad is responsible for the hydrolysis of peptide bonds, and the sequences surrounding the three residues are highly conserved in serine proteinases. For hydrolysis of a substrate to occur, a close interaction of the protein with the catalytic triad of the proteinase is needed. The amino acids that make up the electron-shuttling system provide in essence the machinery that leads to covalent modification of a substrate, as shown in Figure 2. The hydrolysis of the peptide bond starts when the hydroxyl group of the serine residue (of the catalytic triad) is sufficiently close to the carbonyl carbon of the peptide bond to be cleaved. Following transfer of the hydroxyl proton to the nearby histidine residue, the hydroxyl group attacks the carbonyl group, forming a tetrahedral high-energy intermediate in which the carbonyl oxygen atom assumes a negative charge. This oxyanion is stabilized by hydrogen-bonding interactions with the amino groups of Ser 195 and Gly 193. The tetrahedral intermediate collapses and releases the amine product, leaving behind the acyl enzyme. Incoming water forms a second short-lived tetrahedral intermediate, which upon decomposition releases the N-terminal acid product and the free enzyme.

The active sites of proteinases not only contain the catalytic site but also specific binding sites on the enzyme surface, which interact noncovalently with individual amino acid residues of the polypeptide substrate. The peptide bond hydrolyzed by the proteinase is called the scissile or the P1-P1′ bond (Fig. 3). The amino acids extending toward the N-terminus of the substrate are referred to as P1, P2, P3, etc., while those residues on the C-terminal side of the

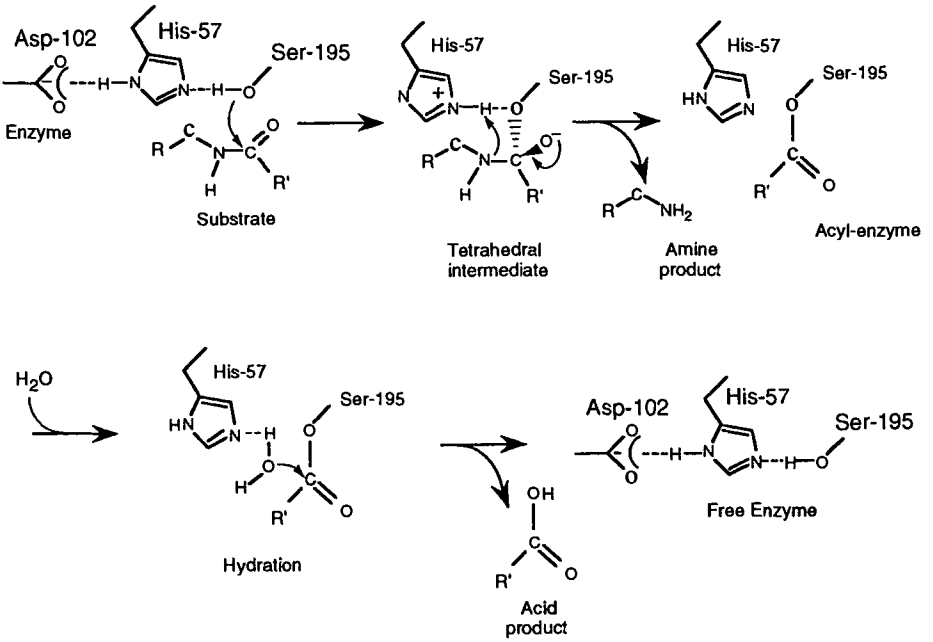

Figure 2 Mechanism of peptide bond hydrolysis by serine proteinases.

scissile bond are designated as P1′, P2′, P3′, etc. The substrate interacts with specific corresponding subsites on the proteinase, which are referred to as S1, S1′, etc. [20]. The subsites on the enzyme may be single amino acid residues, however, more commonly they consist of several amino acid residues, which may even stem from different loops of the enzyme to form a binding pocket. Differences in the amino acid residues that make up the binding pocket ultimately determine the substrate specificity among different serine proteinases.

Even though serine proteinases share a common mechanism of peptide bond cleavage, they differ dramatically in their primary substrate specificity, exhibiting a preference for a certain type of amino acid residue. Hydrolysis studies using natural and synthetic substrates have demonstrated that NE preferentially cleaves peptide bonds after small aliphatic amino acid residues such as valine and alanine [21]. Elastase has been shown to have a pH optimum between 8.0 and 9.0 with most protein substrates and is strongly inhibited by the plasma inhibitor α_1-PI.

NE is a glycoprotein composed of a single polypeptide chain consisting of 218 amino acid residues [22]. The amino acid sequence of NE was determined by standard protein sequencing and has been confirmed by DNA sequencing [23,24].

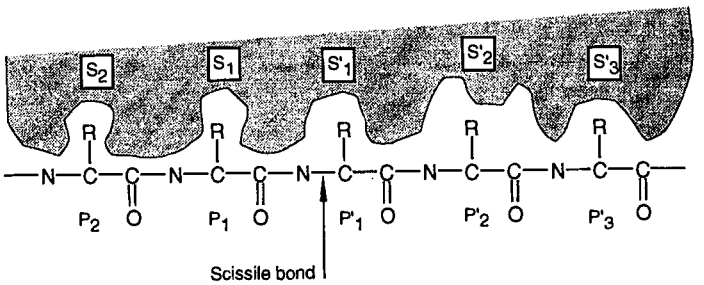

Figure 3 Interaction of amino acid residues on the substrate, which are designated P2, P1, P1′, P2′, with specific subsites on the enzyme S2, S1, S1′, S2, which make up the extended binding region.

NE is a basic protein due to the large number of arginine residues and has an isoelectric point between 10 and 11 [25]. There are at least three iso-forms of NE, which can be separated by isoelectric focusing or by polyacrylamide gel electrophoresis. The isoforms have identical N-terminal sequences and similar catalytic activity and are believed to arise from minor differences in two N-linked carbohydrate side chains. The major form contains about 22% carbohydrate.

The name elastase would suggest that the degradation of elastin is the main function of NE. It is true that NE degrades elastin, however, the enzyme is able to cleave many other structural and soluble proteins. For example, NE cleaves collagen types I–IV, fibronectin, cartilage proteoglycans, laminin, and structural proteins of the glomerular basement membrane [1,3,26–28]. NE also cleaves several plasma proteins such as proteinase inhibitors, components of the complement system, and immunoglobulins. It also has the ability to digest some bacterial and viral proteins [4,29,30].

B. Cathepsin G and Proteinase 3

In addition to NE, located within the azurophilic granules of neutrophils are two other serine proteases—cathepsin G and protease 3. Cathepsin G is a chymotrypsinlike protease which preferentially cleaves peptides adjacent to bulky aliphatic or aromatic residues [31]. It is present within the granules in about equal quantities as NE and its physiological inhibitor is α_1-antichymotrypsin [5]. Like NE, cathepsin G is a basic glycoprotein consisting of a single polypeptide chain of 224 amino acid residues, which exists in at least four forms as demonstrated by electrophoresis [32,33]. Although the exact function of this proteinase is unknown, its proteolytic and bactericidal activity suggests that cathepsin G participates in the killing and digestion of pathogens engulfed during phagocytosis.

The proteinase is capable of degrading elastin, although at a much slower rate than NE, as well as numerous other protein substrates such as collagen, fibronectin, and laminin [34,35]. The extent to which cathepsin G participates in extracellular degradation is still unclear, however, it has been suggested that cathepsin G acts in a synergistic manner to facilitate the degradation of elastin by NE [34].

Proteinase 3 (PR3) is a potent, nonspecific proteinase, which has a broad substrate specificity including matrix proteins. Structural studies reveal that PR3 has an active site similar to NE, and hydrolysis studies show that the enzyme preferentially attacks peptide bonds adjacent to small aliphatic amino acid residues [36]. PR3 has been shown to degrade elastin, although its elastinolytic activity is about one-half that of NE at physiological pH but it solubilized more elastin than NE at pH 6.5 [37]. Intratracheal instillation of PR3 has been demonstrated to cause emphysemalike changes in hamsters similar to those observed after NE application in a similar animal model [37,38]. The amount of PR3 within the granules is about the same quantity as that of NE and cathepsin G, but the function and role of PR3 in lung pathology remains unknown. The enzyme is not as basic as NE, with an isoelectric point around 8. Its major inhibitor is α_1-PI, but this inhibitor binds NE more readily than PR3 [39]. Interestingly, secretory leukoprotease inhibitor (SLPI) is unable to inhibit PR3. PR3 can degrade a variety of other matrix proteins such as fibronectin, laminin, and collagen type IV [40].

C. Other Neutrophil Proteinases

Neutrophils also contain and secrete two metalloproteinases: collagenase and gelatinase [41–43]. Metalloproteinases differ from serine proteinases in that they utilize a heavy metal (Zn^{2+}) at the catalytic site for activity. These proteinases are stored as inactive proenzymes within secondary and tertiary granules. Collagenase and gelatinase can be activated extracellularly by NE and are capable of degrading collagen, fibronectin, and elastin [44]. These proteinases are active at neutral pH, and their activity within the lung is controlled by tissue inhibitor of metalloproteinases (TIMP). The extent to which these metalloproteinases are involved in extracellular matrix degradation associated with lung damage is still speculative, however, degrading collagen and fibronectin may allow the elastin to be more easily available for NE binding and hydrolysis.

D. Macrophage Elastases

There is no question about the destructive potential of neutrophil proteinases and their ability to degrade extracellular matrices. A topic that has been more

controversial is the ability of macrophages to degrade elastin. This is of relevance because smokers have 5- to 10-fold more macrophages in their lower respiratory tract than nonsmokers, and the number of macrophages outnumber the neutrophils by severalfold. Monocytes contain small amounts of elastase, while macrophages have cell surface receptors that bind and internalize NE, which can later be released in an active form [45]. It has been established that macrophages synthesize and secrete a number of metalloproteinases, which include collagenase, stromelysins, and gelatinase capable of degrading matrix collagen, fibronectin, elastin, laminin, and other extracellular glycoproteins at neutral pH [46,47]. A second class of proteinases produced by macrophages that have elastinolytic activity are the cysteine proteinases. Cathepsins B, L, and S solubilize elastin, however, since these enzymes are most active in acidic environments, they most likely function intracellularly degrading phagocytized particles [48,49].

IV. NATURAL PROTEIN INHIBITORS OF ELASTASE

Proteinases are involved in a variety of biological processes ranging from digestion to coagulation and complement activation. The activity of these proteinases must be regulated by potent and specific inhibitors. Numerous proteinaceous inhibitors that inhibit elastase have been isolated and characterized from both animals and plants. In humans, proteinase inhibitors represent the third largest group of proteins by weight in plasma. Human elastase inhibitors are also produced at specific locations such as cells of the skin and glandular cells of the upper airway. Many variants of these inhibitors have also been produced by recombinant technology.

The proteinase inhibitors reviewed in this chapter consist of a single polypeptide chain, which rapidly combines with its target proteinase to form a stable complex and prevent hydrolysis of substrates. A kinetic mechanism valid for most inhibitors of serine proteinases has been proposed by Laskowsky [50]. In this model, the binding loop of the inhibitor interacts with the active site of the enzyme similar to a substrate. After binding, the inhibitor undergoes a series of conformational alterations. Due to the very rigid conformation of the loop that contains the reactive site, cleavage of the inhibitor proceeds extremely slowly, if at all, and the tight interaction between the proteinase and inhibitor results in an inactivation of the enzyme.

There are two principal ways in which inhibitors arrest the sequence of events normally involved in peptide cleavage:

1. The first tetrahedral intermediate cannot be properly formed, for example, if the distance between the hydroxyl of the serine is not appropriate. The

tight binding of the enzyme-inhibitor complex would then stem solely from multiple noncovalent attachment points on the surface of the enzyme.

2. The second tetrahedral intermediate cannot be formed (for example, by exclusion of water). The resultant acyl enzyme would then be inactive due to the existing covalent bond between enzyme and inhibitor. The acyl enzyme could be further stabilized by attachment of the inhibitor to the enzyme at multiple sites.

A. Human α_1-Proteinase Inhibitor

α_1-Proteinase inhibitor (α_1-PI, also referred to as α_1-antitrypsin) is able to inhibit a variety of serine proteinases, including many trypsin and chymotrypsinlike enzymes, but its major physiological target is NE. α_1-PI is quantitatively the most important molecule involved in forming the antielastase screen in human lung tissue, making up over 90% of the antielastinolytic protection in the lower airways. It is the best characterized member of the serpin (SERine Protease INhibitor) superfamily of proteins. Members of the serpin family include not only proteinase inhibitors such as α_1-antichymotrypsin, antithrombin III, glia-derived nexin [51], but also proteins with transport function (steroid binding protein) and protein carriers of biologically active peptides (angiotensinogen) [52], as well as proteins with less well-characterized functions such as ovalbumin and barley endosperm protein Z [53].

α_1-PI consists of a single glycosylated polypeptide chain with a molecular weight of 52 kDa containing about 20% carbohydrate and no disulfide bonds (Table 2). The bulk of α_1-PI is produced in hepatocytes. The liver synthesizes and secretes approximately 2 g of inhibitor per day into the circulation. As an acute-phase protein, blood levels vary considerably (normal level is approximately 25–50 μmol/L). The reactive center of α_1-PI contains a methionine residue at position P1 [54]. This amino acid is susceptible to oxidation by a variety of oxidizing agents, resulting in a marked decrease in the association rate of the

Table 2 Properties of Proteinaceous Elastase Inhibitors

Inhibitor	MW	Source	P1-P1′	Oxidative sensitive	Proteolytic sensitive
α_1-PI	52,000	Human	Met358-Ser	Yes	Sensitive
SLPI	11,300	Human	Leu72-Met	Yes	Sensitive
Elafin	6,000	Human	Ala23-Met	Predicted	
Eglin c	8,100	Leech	Leu45-Asp	No	Resistant

inhibitor with NE. α_1-PI can be inactivated by biological oxidants generated from the myeloperoxidase enzyme system as well as oxidants in cigarette smoke.

The binding of α_1-PI to NE is very tight, however, whether a covalent bond is formed between enzyme and inhibitor is controversial. Upon binding to human leukocyte elastase, α_1-PI is cleaved slowly between P1 and P1′ (Met-Ser) of the reactive loop rendering an inactive inhibitor. The conformational change due to this cleavage takes the newly formed ends to opposing sides of the molecule [55]. The crystal structure of native α_1-PI is not available, but the clipped form of the inhibitor has been crystallized and its structure determined. In proteolytically modified α_1-PI, the active site loop is integrated into the A-sheet of the molecule to complete the five-stranded β-pleated sheet. At slightly elevated temperatures, the intact α_1-PI reactive loop has an increased tendency to insert into the A-sheet of another α_1-PI molecule [56] and thus shows a disposition to polymerize. This is particularly pronounced in α_1-PI–deficient Z homozygotes (PiZZ) and is believed to be caused by the increased flexibility of the loop due to the Glu342Lys mutation [57]. It is hypothesized that this process of inserting the active site loop of one molecule into the A-sheet of another is the cause for the reduced excretion of α_1-PI from the liver cells in PiZZ individuals (only 15% of the produced α_1-PI is excreted).

The gene encoding α_1-PI has been cloned and the recombinant protein expressed in both yeast and in *E. coli* [58,59]. Recombinant α_1-PI differs from native α_1-PI in that it lacks the carbohydrate side chains and contains N-acetyl-methionine at its N-terminus [60]. Its molecular weight is approximately 42,000 daltons. Since the inhibitory properties of the recombinant α_1-PI against elastase are similar to the wild-type molecule, production of recombinant protein in yeast provides an abundant source of the inhibitor.

Pharmacokinetic studies in several animal species (see Table 3) have shown that nonglycosylated recombinant α_1-PI has a much shorter plasma half-life than the native form of α_1-PI (1–2 hours vs. 2–4 days) [61,62]. Since the proposed mode of delivery of α_1-PI is by aerosolization, rapid plasma elimination should not be a concern. It has been demonstrated that both recombinant and native α_1-PI can be aerosolized without loss of inhibitory activity and animal studies have shown that aerosolized α_1-PI has an extended lung half-life. We have also investigated the pharmacokinetics of recombinant α_1-PI in rat. The distribution and elimination half-life after bolus intravenous application of 2 mg/kg were approximately 25 and 100 minutes, respectively. After intratracheal application of recombinant α_1-PI, plasma levels peaked between 4 and 8 hours after administration, and the half-life of disappearance from the lung was approximately 12.5 hours as measured by ELISA and 11 hours as determined by antielastase activity. This confirms that α_1-PI administered to the lung remains active for an extended period of time and is able to cross from the lung into the blood. Low amounts of

Table 3 Resident Plasma and Lung Half-Time of Inhibitors in Different Species

Inhibitor	Species	Plasma ($t_{1/2}$)	Lung ($t_{1/2}$)	Ref.
α_1-PI	Mouse	2.5 hr		62
	Rabbit	40.0 hr		61
	Human	5.0 days		61
Recombinant α_1-PI	Mouse	12.0 min		62
	Rat	1.5 hr	12.0 hr	[a]
	Rabbit	10.0 hr		[a]
SLPI	Rat	50.0 min	4.5 hr	68
	Human		5.0 hr	82
Eglin c	Rat	35.0 min		96
	Rabbit	35–50 min		96
	Baboon	2–2.5 hr		96

[a]Unpublished data.

recombinant α_1-PI were found in the urine of rats after i.v. bolus injection of 2 mg/kg, in contrast to studies performed in monkeys [63], where approximately 38% of the injected material appeared in the urine within 3 hours after treatment. However, the dose used in the monkey study was 60 times greater than the amounts used in our study. Other investigators have shown in the mouse [64] that recombinant α_1-PI was cleared rapidly from the blood. The available data suggest that recombinant α_1-PI, in analogy to other small proteins, is excreted by glomerular filtration. At low concentrations, tubular resorption and degradation prevent excretion of the unchanged substance in urine.

α_1-PI has been shown to be efficacious against NE-induced emphysema in the hamster when given intratracheally before NE instillation [65]. Plasma purified α_1-PI has been administered by aerosol inhalation (100 mg every 12 hours for 7 days) to 12 homozygous α_1-PI–deficient patients [66]. The treatment appeared to be well tolerated and returned the antielastase defenses in the lower respiratory tract to normal levels. In further studies checking for allergenic responses to nonglycosylated recombinant α_1-PI, the inhibitor was administered by repeated subcutaneous injections and by inhalation to α_1-PI–deficient patients. No hypersensitivity or humoral and cellular reactions were noted and no unwanted effects observed.

The logical patient group to test the benefits of antielastase supplementation are individuals who are genetically α_1-PI deficient (PiZZ). This type of therapy

using partially purified plasma α_1-PI has already been practiced for many years and is pharmacologically effective and clinically accepted. Weekly i.v. applications of 60 mg/kg native α_1-PI to PiZZ patients were sufficient to maintain serum trough levels above the threshold level of 80 mg/dL, which is thought to provide (by diffusion) adequate protection from NE in the lung of PiZZ individuals [67]. Later, it was shown that once-monthly infusions of 250 mg/kg of native α_1-PI also provided adequate protection to the lung [68]. An important finding was the quantity of α_1-PI found in bronchoalveolar lung lavage was more than double the pretreatment level, indicating the inhibitor was able to cross the interstitium and enter the lung. Intravenous administration of α_1-PI is the accepted treatment for α_1-PI–deficient individuals with emphysema, however, its efficacy is difficult to evaluate since no formal controlled studies have been performed.

In a 1-week study of cystic fibrosis patients treated with aerosolized native α_1-PI, it was shown that inhibitor levels reached 8 μmol/L, which was sufficient to completely suppress NE activity in the epithelial lining fluid [69]. Such levels were reached in several patients who were given 1.5–3 mg/kg native α_1-PI every 12 hours. In the same study it was reported that weekly intravenous doses of 120 mg/kg native α_1-PI given over a time period of one month was not sufficient to suppress NE activity in epithelial lining fluid of cystic fibrosis patients. This study proved that α_1-PI can be delivered active as an aerosol to the lung of patients and that concentration levels can be reached that are sufficient to inhibit NE activity.

B. Secretory Leukocyte Proteinase Inhibitor

Secretory leukocyte proteinase inhibitor (SLPI, also called antileukoprotease, bronchial mucous inhibitor, human seminal inhibitor, and mucous proteinase inhibitor) is a potent inhibitor of NE, trypsin, and chymotrypsin [70]. It is found in all mucous secretions and in very low concentrations in the plasma. In the lung, SLPI is synthesized in the central airways by serous glandular cells and in the lower respiratory tract by Clara cells and goblet cells [71,72]. Is believed to be the major antielastase of the upper airways. SLPI consists of a single polypeptide chain of molecular weight 12,000 daltons [73]. It is comprised of 107 amino acid residues, which are organized into two distinct but closely related domains (N-terminal domain, residues 1–54; C-terminal domain, residues 55–107). Each of these domains is stabilized by four disulfide bonds found in identical positions. A striking feature of this molecule is its unusual resistance against denaturation by heat and acid, which is probably due to the presence of the eight disulfide bridges.

Although SLPI can inhibit a broad range of serine proteinases, kinetic studies suggest that its major physiological role is to inhibit neutrophil elastase. SLPI can

also inhibit cathepsin G but is unable to inhibit proteinase 3. SLPI interacts with NE to form a reversible complex by which the inhibitor dissociates uncleaved. This interaction is characterized by a high association rate constant and a very low dissociation rate constant, making SLPI only a slightly less efficient inhibitor of NE than α_1-PI. The active site for protease inhibition (P1-P1′ = Leu72-Met73) is located in the C-terminal domain [74], while the N-terminal domain has no antiprotease activity as originally assumed. As with α_1-PI, the reactive center of SLPI is occupied by a methionine residue which is prone to oxidation, leading to inactivation of the inhibitor.

The gene encoding SLPI has been isolated and the recombinant form of the inhibitor has been expressed in *E. coli* [75]. Recombinant SLPI is identical to the naturally occurring human molecule, an important point when considering the possible antigenicity of recombinant molecules after chronic administration. Recombinant technology not only provides an abundant source of the inhibitor for therapy, but also provides an important tool to further investigate the interaction of SLPI with proteinases by directed mutagenesis. It has been shown that the Met73Leu-mutant displayed similar inhibitory properties while its sensitivity to oxidation was markedly reduced [76]. Expression of only the C-terminal domain of SLPI, called half-SLPI, produced a truncated protein, which was as efficient inhibiting NE and cathepsin G as the native inhibitor. However, not all properties of SLPI were maintained with the half-SLPI, which may indicate additional functions of the protein besides inhibition of proteinases [77,78].

Since recombinant SLPI is identical to the native inhibitor, allergenic reactions are not to be expected with this molecule. A one-month toxicity study after aerosol application of recombinant SLPI in cynomolgus monkeys has been performed with no clinically relevant toxicity observed [7]. Intratracheal pretreatment of hamsters with recombinant SLPI (3 mg) resulted in significant protection against NE-induced emphysema even if the animals were pretreated for as long as 8 hours before elastase instillation [79]. Therefore, recombinant SLPI appears to be a safe and effective inhibitor.

Pharmacokinetic studies with recombinant SLPI in rats show that intravenously applied SLPI (2 mg/kg) is rapidly cleared from blood with an elimination half-time of 50 minutes with only minute amounts of the inhibitor appearing in the urine [80]. After intratracheal application (8.6 mg/kg), recombinant SLPI showed a pulmonary half-life of 4–5 hours as determined by ELISA or by activity assay, indicating minimal metabolism in the lung. About 45% of the intrapulmonary SLPI appeared systemically in the plasma, reaching a maximal plasma level 1–2 hours after application. This demonstrates that intratracheally applied SLPI remains active in the lung for several hours and may thus provide effective protection against proteinase mediated lung damage [80]. In sheep, functional SLPI could be recovered by lung lavage several hours after intratracheal

application. Clearance from the lung was slow, with an elimination half-time of 12 hours [81].

Recombinant SLPI has been aerosolized to individuals with cystic fibrosis, and its effect on the NE burden has been evaluated [82]. Aerosol application of SLPI (100 mg/kg) twice daily for one week reduced active NE levels in airway epithelial lining fluid. However, aerosolization of 50 mg/kg SLPI failed to reduce NE activity probably due to the large amount of free elastase in the epithelial lining fluid of cystic fibrosis patients [82]. The half-life of SLPI in the lung after the first application of 100 mg/kg was approximately 5.5 hours. These data demonstrate that sufficient amounts of aerosolized SLPI can significantly reduce the elastase burden of the lung in cystic fibrosis patients.

C. Elafin

Elafin (also called SKALP) is an acid-stable low molecular weight inhibitor recently isolated from the skin of psoriasis patients [83]. It is composed of 57 amino acid residues and has a molecular weight of approximately 6 kDa. The inhibitor is a basic protein with an isoelectric point around pH 9.7. Amino acid sequence comparison shows 40% homology between elafin and the C-terminal domain of SLPI. Elafin is stabilized by four disulfide bridges located in positions similar to the ones found in the second domain of SLPI, suggesting that the two inhibitors may have comparable tertiary structures. The P1-P1′ positions are occupied by the amino acid residues Ala-Met as compared to Leu-Met in SLPI. In fact, the P1′ to P6′ positions seem to be the most conserved continuous sequence of the molecule. The methionine residue at the reactive center of elafin may be prone to oxidation, leading to decreased effectiveness of the inhibitor.

Like SLPI, elafin is a very potent inhibitor of NE. Elafin interacts with elastase in a reversible manner and retains its inhibitory capacity upon dissociation of the complex. Kinetic data have shown that the association rate constant for the formation of an elafin-elastase complex is pH dependent [84]. This is probably due to the basic properties of the inhibitor and enzyme. Elafin also inhibits proteinase 3 but has no effect on cathepsin G, trypsin, or chymotrypsin. This is interesting in view of the fact that SLPI inhibits cathepsin G but is relatively ineffective against proteinase 3.

The cDNA suggests that elafin is synthesized as a precursor peptide consisting of 117 amino acid residues. This precursor form carries a hydrophobic region of 22 amino acid residues at its N-terminus believed to represent a signal peptide [85]. In addition to the inhibitory domain, the remaining 95-amino-acid-residue peptide contains an N-terminus domain with four repeats, which are homologs to the putative transglutaminase substrate motifs. The authors speculate that elafin can exist either as the free 6 kDa inhibitor or as the immobilized transglutaminase

cross-linked 9.9 kDa form. The precise biological function of elafin is not known, however, its inhibition profile and origin suggests a role in regulation of polymorphonuclear leukocyte–dependent tissue destruction in cutaneous inflammation [86].

Due to the recent discovery of elafin, very little information is available concerning its pharmacokinetic properties and effectiveness in vivo. However, this naturally occurring inhibitor has many properties that make it attractive, such as its size and ability to inhibit proteinase 3 as well as NE and therefore may be of therapeutic value in diseases resulting from uncontrolled elastinolytic activity.

D. Eglin C

Eglin c, from the leech *Hirudo medicinalis,* is a very potent and specific inhibitor of human leukocyte elastase and cathepsin G and was first described by Ursula Seemüller in 1977 [87]. Eglin c consists of a single polypeptide chain of 70 amino acids [88]. Striking features of this molecule are the lack of sulfur-containing amino acids and its outstanding resistance against denaturation by heat, acids, and proteolysis. The loop containing the "scissile" bond with Leu45-Asp46 in the P1-P1′ position is held in place solely by hydrogen bonding [89]. Complex formation with elastase is reversible and does not involve cleavage of the active site bond [90]. The interaction of eglin c and NE is almost as rapid as the interaction of NE with α_1-PI, and the complexes formed are very stable.

The gene for this polypeptide has been chemically synthesized and expressed in *E. coli* [91]. Recombinant eglin c is identical to the native inhibitor, except the N-terminus is blocked by an acetyl group [92]. The expressed inhibitor and the material isolated from the leech have identical inhibitory properties and stability. Eglin c is a selective inhibitor, rapidly inactivating NE, cathepsin G, mast cell chymase, chymotrypsin, and pancreatic elastase, but a weak inhibitor of proteinase 3 while essentially ineffective against other known human proteinases. A double mutant form of eglin c in which Leu45 (P1-position) was replaced by an arginine, and Asp46 by a serine (Leu45Arg, Asp46Ser), was shown to be a substrate of trypsin. This was the consequence of a much higher flexibility of the molecule around the scissile bond. Conversely, in the single mutant Leu45Arg, the hydrogen bonding structure of the wild type was maintained and, due to the introduction of a basic residue at the P1 position, produced an inhibitor with antitrypsin specificity [93].

Eglin c has been extensively studied in a number of pharmacological and toxicological test systems. Eglin c showed effectiveness in preventing NE-inflicted lung damage in hamsters, even if pretreated with the inhibitor as long as 8 hours before the insult [94,95]. Pharmacological investigations proved that eglin c was extremely well tolerated with no unwanted effects observed.

Eglin c did not cause any adverse or toxic effects after a single bolus injection of 400 mg/kg in rabbits and baboons, nor after daily injections of up to 100 mg/kg for 30 days in rabbits and 14 days in baboons [96].

The potential of eglin c to cause allergic reactions was first noted in people who were handling the biomaterial (three individuals who had a history of allergies). Although no adverse effects of eglin c were noted in animals in subchronic toxicity studies, in a later study in dogs, serious allergic reactions (anaphylactic shock) were observed. For these reasons, eglin c has not been clinically investigated and offers little promise as a therapeutic agent.

V. DESIRABLE PROPERTIES OF THERAPEUTIC ELASTASE INHIBITORS

The role of therapeutic inhibitors of elastase is to prevent the degradation of extracellular matrix proteins following the release of elastase from activated neutrophils. To determine which inhibitors of elastase may be of therapeutic value, there are several properties that must be considered. These include rate of proteinase inactivation, enzyme selectivity, size, susceptibility to inactivation, and their potential for antigenicity. Some of these properties are reviewed in the following section.

A. Enzymology of Protein Inhibitors of Elastase

In order to efficiently prevent proteolysis, the inhibitor should fulfill at least two requirements: (1) inhibit the target enzyme in an irreversible or pseudo-irreversible manner and (2) suppress the enzyme activity before significant proteolysis has occurred. From a kinetic viewpoint, the classical example of an irreversible protein inhibitor of NE is α_1-PI. Proteolysis can also be prevented by reversible inhibitors if the in vivo inhibitor concentration is much greater than K_i, the equilibrium constant for inhibition ($[I]_{vivo} >> K_i$), resulting in a pseudo-irreversible behavior.

The second important aspect lies in the delay time of inhibition, which is the time elapsed between the release of NE from the neutrophil and the formation of the enzyme-inhibitor complex. When elastase is released from neutrophils, there is a competition between the substrate and the inhibitor for the binding to the enzyme. The notion of delay time of inhibition was developed by Bieth [97,98], where he defined the delay time of inhibition, dt, as the time necessary to inhibit 99% of the enzyme activity.

Bieth postulated that an inhibitor can play a proteolysis-preventing function only if dt is <1 s. This condition is indeed fulfilled by the endogenous α_1-PI, since

dt is estimated to be 0.2 s in the epithelial lining fluid. Equilibrium dissociation constants (K_i) and on-rate constants (k_{on}) of protein inhibitors of NE are summarized in Table 4.

B. Selectivity

None of the protein inhibitors of NE presented in this chapter are protease specific. They all inhibit more than one protease, but they are protease class specific (Table 5). For example, α_1-PI, SLPI, and eglin c are serine-protease inhibitors and inhibit trypsin, chymotrypsin, and cathepsin G in addition to NE. However, α_1-PI is an inhibitor of neutrophil PR3, whereas SLPI and eglin c are only very weak inhibitors of PR3 [40]. By contrast, elafin, which shares 38% homology with the C-terminal domain of SLPI, does inhibit PR3. A strong selectivity for NE is important to reduce toxicity resulting from interference of the inhibitor with other proteolytic processes.

C. Stability

1. Effect of Oxidation

Activated neutrophils release not only elastase and PR3, but also superoxide anions (O_2^-) and myeloperoxidase. These oxidants are able to react with the residue Met358 present at the active-site of α_1-PI [99], which is then converted into methionine sulfoxide [100]. It has been shown that the k_{on} of α_1-PI chemically oxidized by N-chlorosuccinimide is 2000-fold lower than that of the native inhibitor [101], which means that 2000-fold more substrate breakdown will take place during the time of inhibition by the oxidized species. More recently it has been shown that this methionine residue can be oxidized in vivo where oxidants are released by alveolar macrophages [102,103] and by activated neutrophils [104]. The presence of the oxidative-sensitive methionine residue at the reactive

Table 4 Kinetic Parameters with Neutrophil Elastase

	Native inhibitor		Recombinant inhibitor		
	k_{on} ($M^{-1}s^{-1}$)	Ki (M)	k_{on} ($M^{-1}s^{-1}$)	Ki (M)	Ref.
α_1-PI	1.0×10^7	(Irreversible)	2.0×10^7	(Irreversible)	62,101
SLPI	2.8×10^6	6.3×10^{-11}	2.8×10^6	5.0×10^{-11}	73,127
Elafin	2.1×10^6	2.0×10^{-10}			84
Eglin c	1.4×10^7	8.0×10^{-11}	1.6×10^7	7.5×10^{-11}	87,128

Table 5 Ability of Inhibitors to Inactivate Neutrophil Proteinases

Inhibitor	NE	Cathepsin G	Proteinase-3
α_1-PI	+	+	+
SLPI	+	+	–
Elafin	+	–	+
Eglin c	+	+	±

site of this inhibitor may be important in inhibitor regulation; thus, chronic inhibitor therapy may require a similar mechanism of inactivation.

SLPI also contains a methionine residue in its active site (Leu72-Met73), which makes it sensitive to oxidation. Carp and Janoff [105] showed that SLPI could be inactivated by phagocyte-derived oxidants. It was also shown that oxidant-resistant variants (where one to four of the Met residues were replaced by Leu) were better inhibitors of NE when incubated in the presence of the oxidizing agent cis-platinum(II) diamine dichloride [76]. The kinetic properties of SLPI oxidized at the Met residue in position P1′ demonstrates a 10-fold decrease in the on-rate constant with NE, although this change is less dramatic than in the case of α_1-PI [106].

2. Proteolytic Inactivation

α_1-PI can be inactivated as a result of proteolytic cleavage close to or at its active center. Proteolytic inactivation results from the action of neutrophil metalloproteinases [107,108], macrophage cathepsin L [109], and bacterial proteinases such as *Pseudomonas aeruginosa* [110]. It has recently been reported that SLPI can be cleaved by neutrophil proteinase-3 [111] between residues Ala16 and Gln17. However, the PR3-treated SLPI is still active against NE and cathepsin G. Despite the lack of disulfide bonds, eglin c is a particularly stable polypeptide which is quite resistant to proteolytic inactivation [7].

D. Antigenicity

The development of the leech inhibitor eglin c was stopped after serious allergic reactions were noted [7]. This result clearly showed the inherent disadvantage of using protein inhibitors from a nonhuman source for therapy. The recombinant SLPI molecule is identical to the natural species, whereas the α_1-PI produced by gene technology is the nonglycosylated form of the natural molecule. Therefore,

of the two molecules produced by genetic engineering, recombinant SLPI is the one that best mimics the human endogenous molecule, although after expression a renaturation step is necessary to form the correct disulfide bonds and attain the proper conformation [112].

E. Other Factors Modulating and Activity of Proteinase Inhibitors

1. Situations Where NE is Resistant to Inhibition by α_1-PI

When NE is liberated from the neutrophil in the immediate vicinity of an elastin fiber, elastin will strongly compete with the inhibitors present for interaction with NE. Therefore, a substantial amount of NE can escape inhibition and bind to elastin [113]. It has been shown that about 30% of elastin-bound NE resists α_1-PI inhibition [114,104]. However, the low molecular weight inhibitor SLPI readily inhibits elastin-bound elastase [114].

Pericellular proteolysis in the presence of α_1-PI also can occur when neutrophils are in close contact with insoluble matrix proteins. In this particular situation, molecules whose molecular weight exceeds 25 kDa cannot access NE [115,116]. Here again the low molecular weight SLPI is able to inhibit NE-mediated proteolysis where α_1-PI is ineffective in a similar situation [116]. It therefore appears that low molecular weight inhibitors (MW < 25 kDa, i.e., SLPI, eglin c, elafin) will have an advantage over high molecular inhibitors of NE such as α_1-PI essentially because they can better diffuse through the extracellular matrix components.

2. Modulation of the Inhibitory Activity by Glycosaminoglycans

Neutrophil elastase is a basic molecule (isoelectric point > 10) that forms tight complexes with a variety of polyanions such as chondroitin sulfate [117], glycosaminoglycan-peptide [118], pentosan polysulfate [119], glycosaminoglycane polysulfate [120], and heparin [121,122]. The nature of the interactions is most likely electrostatic and probably involves some of the 19 arginine residues that are located in clusters on the surface of the enzyme [123,124]. The net result of these interactions between NE and negatively charged molecules is a partial inhibition of the enzyme. This modulation of the activity of α_1-PI/NE interaction by glycosaminoglycans might have some physiological bearings, since it is known that the azurophilic granules contain negatively charged proteoglycans together with the positively charged NE [124]. It is therefore probable that NE is released from the neutrophil in a complex with these ligands. In addition, lung mast cells that secrete heparin [125] are found in large quantities in the alveolar walls [126].

Although α_1-PI does not interact with heparin, SLPI has been shown to tightly bind to this sulfated glycosaminoglycan [127]. Moreover, it has been shown that heparin strongly enhances the rate of NE inhibition by SLPI in vitro. This heparin-activation phenomenon is not observed with elafin, which resembles the C-terminal domain of SLPI [84]. The N-terminal domain of SLPI could thus play a role different from a direct inhibitory action by interacting with negatively charged molecules, thus modulating the activity of the inhibitor.

Recent clinical trials revealed that relatively high concentrations of aerosolized SLPI (100 mg/kg twice daily) are necessary to totally suppress NE activity [78]. A possible explanation for this is that SLPI is unable to freely diffuse through the lung due to the presence of negatively charged glycosaminoglycans at the surface of the epithelium, which could "trap" the inhibitor via electrostatic interactions.

VI. CONCLUSIONS

A disruption of the delicate balance between proteinases and their controlling inhibitors is believed to be responsible for the development and progression of several lung diseases such as emphysema. There is an abundance of evidence implicating neutrophil elastase as the major destructive enzyme in these pathogenic conditions. Administration of proteinaceous inhibitors to augment the normal endogenous levels is a feasible therapeutic approach. It would be naive, however, to assume that neutrophil elastase is the only factor responsible for the development of these diseases. In order to validate the proteinase inhibitor augmentation concept and to show clinically beneficial effects, long-term, multicenter, placebo-controlled clinical trials will be required.

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14

Good Standardization Practices

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I. INTRODUCTION

Enzymes have high specificities, and a manifestation of activity can be used as an identification test. If necessary, a second substrate may be used to prove the presence of a particular enzyme. Also, specific inhibition may be applied to either suppress the activity of the enzyme to be determined or to suppress the activity of contaminating enzymes. Enzyme activity is determined by the quantity of substrate transformed or product formed per time unit.

During the measurement of the enzyme reaction, the reaction velocity ideally should remain constant. Case of proteases or hydrolases are known where the reaction rate gradually decreases as a result of an inhibitory effect of the reaction products. Therefore it is recommended that an enzyme assay be based, when feasible, upon a measurement of the initial reaction rate. This initial reaction rate can in most cases be obtained by extrapolation, a minimum reaction time being required for obtaining a sufficiently precise titration of the molecules removed or produced during this fixed time span.

The reaction rate that is measured depends on a number of experimental conditions such as temperature, pH, ionic strength, and the presence or absence of inhibitors or activators. In actual assays one usually chooses conditions ensuring maximal reaction rate. It is only under the conditions specified in the prescribed assay procedure that enzyme units are defined. Whenever different conditions are used, the rate measured does not have a well-defined relationship to the potency of the enzyme preparation under study.

The kind of substrate used must give a relevant specificity to the reaction catalyzed, and this may influence the reaction rate itself as well as the optimal pH value and ionic strength. Whenever possible the substrate should be a pure, well-defined chemical entity. This also means that for a quantitative assay of enzyme activity, the highest substrate purity should be required as well as precise control of the external factors.

The concentration of the substrate should be, wherever possible, sufficient for saturation of the enzyme, so that the kinetics in the standard assay approach zero order with regard to substrate. Should this be impossible because of the poor solubility of the substrate or at a low affinity of the enzyme for the substrate, it would be advisable to determine the Michaelis-Menten constant so that the observed values of the reaction rate can be converted into those that would be obtained on saturation with the substrate.

When the enzyme is itself a pure chemical entity and the only catalytic agent present, which is exceptional, conditions are fulfilled for an unambiguous definition of units of activity, which is proportional to the reaction rate obtained. Furthermore it is essential to have a detailed knowledge of the biochemical transformation that is obtained and its reaction scheme. When the enzyme is not 100% pure, in order to define activity, a reference standard must be used with the same assay method and substrate; the activity of the unknown enzyme preparation is then expressed in terms of the reference standard.

The current state of the art in clinical enzyme standardization has been extensively reviewed by Bergmeyer, and the information on various international organizations is compiled in the series *Methods of Enzymatic Analysis*. The World Health Organization (WHO) and the pharmacopeias on both national and international levels have to some extent been involved in enzyme standardization. A well-known example is the establishment of the international standard for hyaluronidase by WHO in cooperation with the Hyaluronidase Panel of the United States Pharmacopeia and the manufacturers in the United Kingdom, the United States, and France.

The International Union of Biochemistry (IUB) was officially founded in 1953 by an initiative of the British Biochemical Society. At this time enzyme standardization was in a chaotic state, owing to the multiplicity of arbitrarily defined units of enzyme activity and the ill-defined nomenclature. In 1955, the IUB International Commission on Enzymes was created. This led to an improved enzyme nomenclature, which has been used since 1961, and the definition of the International Unit (I.U.) of enzyme activity.

The I.U. is defined as "the amount that catalyzes the transformation of one micromole of substrate per minute, or where more than one bond of a complex substrate is attacked, one microequivalent of the group concerned per minute, under well defined external conditions." The conditions should be optimized for

each assay to obtain maximal initial reaction rates, i.e., optimal substrate concentration, optimal pH value and ionic strength, presence of activators, and absence of known inhibitors. The recommended temperature is 25°C.

The question of the enzyme units was reexamined by the commission of clinical chemistry of the I.U.P.A.C. The recommendations made appeared in a monograph of R. Dybkaer and K. Jörgensen, "Quantities and Units in Clinical Chemistry, Copenhagen 1967." Some formal imperfections of the international I.U.B. definitions were indicated. Objections were made that the minute is not an S.I. unit. It was proposed to define enzyme activity by a catalytic amount (katal) of a system that catalyzes exactly as many cycles per second of a reaction scheme as there are atoms in 0.012 kg of the pure nuclide ^{12}C. This point of view was adopted and recommended by the commission of Biochemical Nomenclature of I.U.B. in 1972, as appearing in "Nomenclature and Classification of Enzymes," together with their units and symbols (revision and extension of recommendations of 1962 and 1964, Elsevier, Amsterdam, 1972). The katal was proposed as a unit of enzymic activity to be used instead of the earlier unit, and this recommendation was approved by the general assembly of the I.U.B. Congress at Stockholm, July 1973.

The recommendations of the I.U.B. were as follows:

1. The quantity of enzyme be measured as catalytic amount.
2. The concept of enzyme unit as a physically undefined "amount of enzyme" be abandoned.
3. Enzymic activities be defined as the rate of reaction of substrate that may be attributed to catalysis by an enzyme.
4. The corresponding unit be the katal (symbolized kat), defined as the catalytic amount of the enzyme that causes a reaction rate of 1 mol/s in an assay system.
5. The derived kind of quantity, catalytic concentration, be defined as the catalytic amount of the enzyme divided by the volume of the system (derived unit = kat/l). (The liter is the S.I. unit, but ordinarily the mL is used.)
6. The derived kind of quantity, specific catalytic amount, be defined as the catalytic amount of the enzyme divided by its mass (derived unit = kat/kg). Ordinarily specific activity is expressed per mg.
7. Derived kind of quantity, molar catalytic amount, be defined as the catalytic amount of the enzyme defined by its amount of substrate (derived unit = kat/mol). Ordinarily molar activity is expressed per micromole (or nanomole) of preparation.

It is realized that a catalytic amount of one katal, corresponding to the reaction of one mole of substrate per second, will often be too large for practical use. In most cases more appropriate numbers will be obtained by using the microkatal (μkat),

nanokatal (nkat), or picokatal (pkat) corresponding to reaction rate of micromoles, nanomoles, or picomoles per second, respectively.

Since the same basic principle underlies both the katal and the I.U., interconversion is possible:

1 kat $= 6.10^7$ I.U.
1 μkat = 60 I.U.
1 nkat = 0.06 I.U.
1 I.U. = 1/60 μkat = 0.01667 μkat = 16.67 nkat

It was assumed that the katal, being related to the second as unit of time, fits better with rate constants of reaction used in chemical kinetics than does the former enzyme unit, since reaction rates are commonly expressed in moles (or appropriate submultiple thereof) per second.

II. STANDARDIZATION OF PHARMACEUTICAL ENZYMES

In 1960, at the general assembly of the International Pharmaceutical Federation (FIP), the obsolescence of various national pharmacopeial methods for assaying pharmaceutical enzymes was demonstrated. An international commission on pharmaceutical enzymes was created to deal with this unsatisfactory situation and develop improved assay methods and guidelines for the preparation of pharmaceutical enzyme reference materials. The Center for Standards has a coordination function in organizing collaborative enzyme assays between academic, industrial, and national pharmaceutical control laboratories and in distributing FIP pharmaceutical enzyme standards. Since 1960, many FIP assay methods and standard preparations have been adopted by national and international pharmacopeias, such as the European Pharmacopoeia. The ultimate goal is to provide official, preferentially nonempirical, standardized assay methods by which comparison of commercially available pharmaceutical enzymes is made possible. The most desirable situation would be an international uniformity of enzyme standards and assay methods, which would allow physicians and clinicians to unambiguously compare the potencies of commercially available enzyme products.

An important aspect of pharmaceutical enzymes is the choice of an appropriate substrate and its appropriate concentration. The substrate should correspond to the specificity of the enzyme and should be relevant to the physiological activity, as far as possible. Fortunately, in some cases the substrate and conditions of the assay are closely related to the situation in which the enzyme is thought to act therapeutically. However, the precise quantification of enzyme activity in a reproducible manner, which is the main purpose in pharmaceutical control, is often difficult to reconcile with such conditions. Thus, the fibrin plate method

may be an excellent device for research on plasmin or plasminogen activators, giving relatively good results, but it proves hard to reproduce in interlaboratory tests.

A fibrin clot containing adsorbed plasmin inhibitors is difficult to define in a chemical or physical sense. Generally, when enzyme reactions occur at surfaces, the porosities and adsorption properties of which are variable, the reproducibility of enzyme assay methods is questionable. The proteinases, to which belong the most important pharmaceutical enzymes, may present some difficulties when natural substrates (proteins) are prescribed. Here, the application of a parallel run with a reference standard is recommended.

For historical reasons many pharmaceutical enzymes are assayed with physiological or biopolymeric substrates (proteins, polysaccharides, bacteria, oil emulsions), which causes a number of theoretical and practical problems. The interpretation of results is difficult when natural substrates are converted into products that are substrates themselves for the next enzymatic attack. Reaction rates often depend on the position of the scissile bonds in the molecule and the chemical nature of the moieties. Hydrolysis can proceed simultaneously on various bonds at various rates. In proteolysis it is assumed that some products are liberated only after denaturation and that during the reaction course new peptide bonds become accessible for hydrolysis. In these cases the enzymatic mechanisms become exceedingly complex, kinetic parameters are apparent values, and experimental results are strongly influenced by the reaction conditions. Reproducibility problems can occur upon assaying proteinases with a limited specificity for particular casein types. Bromelain and pancreatic proteinase, FIP pharmaceutical enzyme standards, are assayed with a casein substrate. The extent of soluble peptide release is a measure of proteolytic activity. However, due to limited specificity, some proteinases release peptides with a nonrandom aromatic amino acid composition. Contamination of casein preparations with protein and of test enzyme substances with other proteinases biases the assay results. Under these conditions, relative assay methods are indicated.

Lipases are a particular group of esterases acting specifically on insoluble esters at lipid/water interfaces. The physical-chemical properties of substrate emulsions determine the kinetic parameters of the system, and emulsions with identical water:lipid ratios may have totally different degrees of dispersion and therefore a different interfacial surface, which is a true measure of substrate concentration. Cofactors and detergents at the emulsion interface complicate the determination of the true interfacial area and also influence the lipolysis rate. A decrease of lipolytic activity caused by enzyme denaturation and product inhibition by long-chain soaps can be counteracted by adding surface-active agents to lower the interface energy level and albumin to bind inhibitory fatty

acids, respectively. Colipase adsorbs onto the substrate interface through its tyrosyl residue–containing binding site. It anchors lipase to the interface and reduces the lag phase observed in emulsion systems of long-chain triglycerides stabilized by phospholipids. Colipases are species specific to a certain extent. For example, canine colipase stimulates the lipolytic activities of lipases isolated from canine, bovine, and porcine pancreas but not of rat, human, or fungal origin. The former have almost identical isoelectric points, unlike the latter group. The interface recognition site of pancreas lipase involves serine and γ-carboxyl residues, derivatization of which abolishes lipase but not esterase activity.

Lysozyme hydrolyzes bacterial cell-wall proteoglycan, which is readily accessible in gram-positive bacteria and protected by lipids in gram-negative organisms. Its assay is based upon the rate of clearance of a *Micrococcus luteus* suspension. Obviously stringent requirements for preparing the substrate must be met. Suspension characteristics (aggregates of bacteria), batch-to-batch variations (cell wall thickness dependent on harvesting time relative to growth phase), and the pH and ionic strength of the suspension buffer (electrostatic interaction between lysozyme and negatively charged proteoglycan) strongly influence the lysis rate. Standardization of substrate manipulation and assaying relative to a known standard with one single substrate batch make the turbidimetric assay a valuable tool. It is even possible to use the bacterial substrate in the analysis of the dependence of the effective pK of the catalytic residues in the enzyme on the ionic strength and the protein charge according to a modified Debye-Hückel model.

Digestive enzyme preparations often contain *Aspergillus niger* cellulase, which is an enzyme complex consisting of various endo- and exo-*b*-1,4-glucanases, β-glucosidase, cellobiophydrolases, etc. Assays of reducing sugar lack the sensitivity and specificity for endocellulase determination since these enzymes cut the cellulose chains randomly with relatively little release of reducing sugars. Viscosimetric methods are more appropriate for endocellulase assay. In contrast, exocellulase activity causes a fast increase of reducing sugars with little change in the weight average chain length. A stable FIP *Aspergillus niger* endocellulase standard is available. Its activity is determined with a viscometric assay on a hydroxyethylcellulose (HEC) substrate standard with known polymeric properties. The kinetics of this process were investigated by means of static low-angle-laser light scattering (LALLS), which allows continuous monitoring of the decrease of the weight average molecular weight, M_W. These data are converted to number average (M_r) molecular weight values in order to obtain the number of bonds hydrolyzed as a function of time.

The elucidation of enzyme specificity triggered the development of synthetic substrates. These are soluble and chemically well-defined molecules, allowing easy-to-perform assays, straightforward quality control, and kinetic

characterization of the enzyme activity in terms of catalytic events (one catalytic event equals one single substrate molecule undergoing catalysis). A wide variety of synthetic substrates ranging from relatively low to very high selectivity is commercially available. These substrates typically consist of a portion recognized by the enzyme (peptide or saccharide residue) and a moiety released by hydrolysis and detected by titration or monitoring of its spectral properties.

However, large physiological substrates offer more subsite interaction possibilities than small synthetic ones and hence are more selective for particular enzymes. In this respect synthetic substrates provide less information on structural features and binding sites. Some tripeptide chromogenic-substrate hydrolysis products are competitive inhibitors of particular serine proteinases.

Expression of activity in units based on molar concentrations is only possible when the substrate molar concentration, the reaction mechanism, and the resulting rate equation are known.

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Assays

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This chapter contains the assays relevant to the measurement of enzymatic activities based upon methods controlled regularly by collaborative assays and for which enzyme standards are currently available.

Eur. Pharm. following an assay title indicates that the unit of the European Pharmacopoeia, the method, and the standard (B.R.P.: Biological Reference Product) are exactly equal to the F.I.P. unit, method, and standard. The standard and the complete monograph containing the characteristics, identification, and tests can be obtained from the European Pharmacopoeia Secretariat, 226 Route de Colmar F67100, Strasbourg, France. Council of Europe: Telephone [+33]3 88 41 20 00; Telefax [+33]3 88 41 27 71.

F.I.P. following a title means that the European Pharmacopoeia has not published a monograph concerning this enzyme. Standards and further information on such enzymes can be obtained from the Centre for Standards F.I.P., Harelbekestraat 72, B-9000 Ghent, Belgium (Telephone [+32]9 264 80 47; Telefax [+32]9 264 81 89).

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I. PANCREATIS PULVIS (Eur. Pharm.; F.I.P.)

Pancreas powder is prepared from the fresh or frozen pancreases of mammals. It contains various enzymes having proteolytic, lipolytic, and amylolytic activities. One milligram of pancreas powder contains not less than 1.0 European

Pharmacopoeia Unit (Ph. Eur. U.) of total proteolytic activity, 15 European Pharmacopoeia Units of lipolytic activity, and 12 European Pharmacopoeia Units of amylolytic activity. Pancreas powder is prepared under conditions designed to minimize microbial contamination.*

A. Total Proteolytic Activity

The total proteolytic activity of pancreas powder is determined by comparing the quantity of peptides nonprecipitable by a 5% m/V solution of trichloroacetic acid R released per minute from a substrate of casein solution with the quantity of such peptides released by pancreas powder (protease) BRP from the same substrate in the same conditions. For the test suspension and the reference suspension, prepare the suspension and carry out the dilution at 0–4°C.

1. Test Suspension

Triturate 0.100 g of the substance to be examined for 5 min adding gradually 25 mL of 0.02 M calcium chloride R. Transfer completely to a volumetric flask and dilute to 100.0 mL with 0.02 M calcium chloride R. To 10.0 mL of this suspension add 10.0 mL of enterokinase solution and heat in a water bath at 35 ± 0.05°C for 15 min.

Cool and dilute with borate buffer solution pH 7.5 R at 5 ±3 °C to a final concentration of about 0.065 Ph. Eur. U. of total proteolytic activity per milliliter calculated on the basis of the stated activity.

2. Reference Suspension

Prepare a suspension of pancreas powder (protease) BRP as described for the test suspension but without addition of enterokinase so as to obtain a known final concentration of about 0.065 Ph. Eur. U. per milliliter calculated on the basis of the stated activity.

Designate tubes in duplicate T, T_b, S_1, S_{1b}, S_2, S_{2b}, S_3, S_{3b}; designate a tube B.

Add borate buffer solution pH 7.5 R to the tubes as follows:

B: 3.0 mL
S_1 and S_{1b}: 2.0 mL
S_2, S_{2b}, T, and T_b: 1.0 mL

*National authorities may require that pancreas powder comply with a limit for total viable count of 10^4 microorganisms per gram, determined by plate count. National authorities may require that pancreas powder comply with the tests for *Escherichia coli* and *Salmonella*.

Add the reference suspension to the tubes as follows:

S_1 and S_{1b}: 1.0 mL
S_2 and S_{2b}: 2.0 mL
S_3 and S_{3b}: 3.0 mL

Add 2.0 mL of the test suspension to tubes T and T_b.

Add 5.0 mL of a 5.0% m/V solution of trichloroacetic acid R to tubes B, S_{1b}, S_{2b}, S_{3b}, and T_b. Mix by shaking.

Place the tubes and the casein solution in a water bath at 35 ± 0.5°C.

Place a glass rod in each tube. When temperature equilibrium is reached, add 2.0 mL of the casein solution to tubes B, S_{1b}, S_{2b}, S_{3b}, and T_b. Mix.

At time zero, add 2.0 mL of casein solution successively and at intervals of 30 s to tubes S_1, S_2, S_3, and T. Mix immediately after each addition.

Exactly 30 min after addition of the casein solution, taking into account the regular interval adopted, add 5.0 mL of a 5.0% m/V solution of trichloroacetic acid R to tubes S_1, S_2, S_3, and T. Mix. Withdraw the tubes from the water bath and allow to stand at room temperature for 20 min.

Filter the contents of each tube twice through the same suitable filter paper* previously washed with a 5.0% m/V solution of trichloroacetic acid R, then with water and dried.

A schematic presentation of the above operations is shown in the following table:

	Tubes								
	S_1	S_{1b}	S_2	S_{2b}	S_3	S_{3b}	T	T_b	B
Buffer solution	2	2	1	1			1	1	3
Reference suspension	1	1	2	2	3	3			
Test suspension							2	2	
Trichloroacetic acid solution		5		5		5		5	5
Mix		+		+		+		+	+
Water bath, 35°C	+	+	+	+	+	+	+	+	+
Casein solution		2		2		2		2	2
Mix		+		+		+		+	+
Casein solution	2		2		2		2		
Mix	+		+		+		+		
Water bath, 35°C, 30 min	+	+	+	+	+	+	+	+	+
Trichloroacetic acid solution	5		5		5		5		
Mix	+		+		+		+		
Room temperature, 20 min	+	+	+	+	+	+	+	+	+
Filter	+	+	+	+	+	+	+	+	+

* A suitable filter paper complies with the following test: filter 5 mL of a 5.0% solution of trichloroacetic acid R on a 7-cm disc of white filter paper; the absorbance of the filtrate, measured at 275 nm using unfiltered trichloroacetic acid solution as the compensation liquid, is less than 0.04.

Measure the absorbance of the filtrates at 275 nm using the filtrate obtained from tube B as the compensation liquid.

Correct the average absorbance values for the filtrates obtained from tubes S_1, S_2, and S_3 by subtracting the average values obtained for the filtrates from tubes S_{1b}, S_{2b}, and S_{3b}, respectively. Draw a calibration curve of the corrected values against volume of reference suspension used.

Determine the activity of the substance to be examined using the corrected absorbance for the test suspension ($T-T_b$) and the calibration curve, taking into account the dilution factors.

The test is not valid unless the corrected absorbance values are between 0.15 and 0.60.

B. Lipolytic Activity

The lipolytic activity is determined by comparing the rate at which a suspension of pancreas powder hydrolyzes a substrate of olive oil emulsion with the rate at which a suspension of pancreas powder (amylase and lipase) BRP hydrolyzes the same substrate under the same conditions. The test is carried out under nitrogen.

1. Apparatus

Use a reaction vessel of about 50 mL capacity provided with:

A device that will maintain a temperature of 37 ± 0.5°C
A magnetic stirrer
A lid with holes for the insertion of electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of reagents

An automatic or manual titration apparatus may be used. In the latter case, the burette is graduated in 0.005-mL increments and the pH meter is provided with a wide reaching scale and glass-calomel electrodes. After each test the reaction vessel is evacuated by suction and washed several times with water, the washings being removed each time by suction.

2. Test Suspension

In a small mortar cooled to 0°–4°C, triturate carefully a quantity of the substance to be examined equivalent to about 2500 Ph. Eur. U. of lipolytic activity with 1 mL of cooled maleate buffer solution pH 7.0 R (lipase solvent) until a very fine suspension is obtained. Dilute the suspension with cold maleate buffer solution pH 7.0 R, transfer quantitatively to a volumetric flask, and dilute to 100.0 mL with the cold buffer solution. Keep the flask containing the test suspension in iced water during the titration.

3. Reference Suspension

To avoid absorption of water formed by condensation, allow the reference preparation to reach room temperature before opening the container.

Prepare a suspension of pancreas powder (amylase and lipase) BRP as described for the test suspension using a quantity equivalent to about 2500 Ph. Eur. U.

Carry out the titrations immediately after preparation of the test suspension and the reference suspension. Place 29.5 mL of olive oil emulsion in the reaction vessel equilibrated at 37 ± 0.5°C. Fit the vessel with the electrodes, a stirrer, and the burette (the tip being immersed in the olive oil emulsion).

Put the lid in place and switch on the apparatus. Carefully add 0.1 N sodium hydroxide with stirring to adjust to pH 9.2. Using a rapid-flow graduated pipette, transfer about 0.5 mL of the previously homogenized reference suspension, start the chronometer, and add continuously 0.1 N sodium hydroxide to maintain the pH at 9.0. After exactly 1 min, note the volume of 0.1 N sodium hydroxide used. Carry out the measurement a further four times. Discard the first reading and determine the average of the four others (S_1). Make two further determinations (S_2 and S_3). Calculate the average of the values, S_1, S_2, and S_3.

The average volume of 0.1 N sodium hydroxide used should be about 0.12 mL/min with limits of 0.08–0.16 mL.

Carry out three determinations in the same manner for the test suspension (T_1, T_2, and T_3). If the quantity of 0.1 N sodium hydroxide used is outside the limits 0.08–0.16 mL/min, the assay should be started again with a quantity of test suspension that is more suitable but situated between 0.4 and 0.6 mL. Otherwise the quantity of the substance to be examined should be adjusted to comply with the conditions of the test.

Calculate the average of the values T_1, T_2, and T_3.

Calculate the activity in Ph. Eur. Units per milligram from the expression:

$$\frac{n \times m_1}{n_1 \times m} \times A$$

where:

- n = average volume of 0.1 N sodium hydroxide used per minute during the titration of the test suspension
- n_1 = average volume of 0.1 N sodium hydroxide used per minute during the titration of the reference suspension
- m = mass in milligrams of the substance to be examined
- m_1 = mass in milligrams of the reference preparation
- A = activity of pancreas powder (amylase and lipase) BRP in Ph. Eur. Units per milligram

C. Amylolytic Activity

The amylolytic activity is determined by comparing the rate at which a suspension of pancreas powder hydrolyzes a substrate of starch solution with the rate at which a suspension of pancreas powder (amylase and lipase) BRP hydrolyzes the same substrate under the same conditions.

1. Test Suspension

Triturate a quantity of the substance to be examined equivalent to about 1500 Ph. Eur. U. of amylolytic activity with 60 mL of phosphate buffer solution pH 6.8 R1 for 15 min. Transfer quantitatively to a volumetric flask and dilute to 100.0 mL with phosphate buffer solution pH 6.8 R1.

2. Reference Suspension

Prepare a suspension of pancreas powder (amylase and lipase) BRP as described for the test suspension, using a quantity equivalent to about 1500 Ph. Eur. U.

In a test tube 200 mm long and 22 mm in diameter fitted with a ground glass stopper, place 25.0 mL of starch solution, 10.0 mL of phosphate buffer solution pH 6.8 R1, and 1.0 mL of a 1.17% m/V solution of sodium chloride R. Close the tube, shake, and place in a water bath at 25.0 ± 0.1°C. When the temperature equilibrium has been reached, add 1.0 mL of the test suspension and start the chronometer. Mix and place the tube in the water bath. After exactly 10 min, add 2 mL of 1 N hydrochloric acid. Transfer the mixture quantitatively to a 300-mL conical flask fitted with a ground-glass stopper. While shaking continuously, add 10.0 mL of 0.1 N iodine and, immediately thereafter, 45 mL of 0.1 N sodium hydroxide. Allow to stand in the dark at a temperature between 15 and 25°C for 15 min. Add 4 mL of a mixture of four volumes of water and one volume of sulfuric acid R. Titrate the excess of iodine with 0.1 N sodium thiosulfate using a microburette. Carry out a blank titration adding the 2 mL of 1 N hydrochloric acid before introducing the test suspension. Carry out the titration of the reference suspension in the same manner.

Calculate the amylolytic activity in Ph. Eur. Units per milligram from the expression:

$$\frac{(n' - n)\, m_1}{(n'_1 - n_1)\, m} \times A$$

where:

n = number of milliliters of 0.1 N sodium thiosulfate used in the titration of the test suspension

n_1 = number of milliliters of 0.1 N sodium thiosulfate used in the titration of the reference suspension
n' = number of milliliters of 0.1 N sodium thiosulfate used in the blank titration of the test suspension
n'_1 = number of milliliters of 0.1 N sodium thiosulfate used in the blank titration of the reference suspension
m = mass in milligrams of the substance to be examined
m_1 = mass in milligrams of the reference preparation
A = activity of pancreas powder (amylase and lipase) BRP in Ph. Eur. Units per milligram

D. Reagents and Solutions

1. Acacia Solutions

Dissolve 100 g of acacia in 1000 mL of water. Stir with a mechanical stirrer for 2 h. Centrifuge at about 2000 g_n for 30 min to obtain a clear solution.

Store in polyethylene containers with about 250 mL capacity at 0 to –20°C.

2. Buffer (borate)

Buffer (borate) solution pH 7.5. Dissolve 2.5 g of sodium chloride R, 2.85 g of disodium tetraborate R, and 10.5 g of boric acid R in water and dilute to 1000.0 mL with the same solvent. Adjust the pH if necessary.

Store at 2–8°C.

3. Buffer (maleate)

Buffer (maleate) solution pH 7.0. Dissolve 10 g of sodium chloride R, 6.06 g of tris(hydroxymethyl)aminomethane R, and 4.90 g of maleic anhydride R in 900 mL of water. Adjust to pH 7.0 using a 17% m/V solution of sodium hydroxide R. Dilute to 1000 mL with water.

Store at 2–8°C and use within 3 days.

4. Buffer (phosphate)

Buffer (phosphate) solution pH 6.8 R1. To 51.0 mL of a 2.72% m/V solution of potassium dihydrogen phosphate R (KH_2PO_4) add 49.0 mL of a 7.16% m/V solution of disodium hydrogen phosphate R ($Na_2HPO_4 \cdot 12H_2O$). Adjust the pH if necessary.

Store at 2–8°C.

5. Calcium Chloride Solution

Calcium chloride solution 0.02 M. Dissolve 2.94 g of calcium chloride R in 900 mL of water, adjust to pH 6.0–6.2, and dilute to 1000.0 mL with water.

Store at 2–8°C.

6. Casein Solution

Suspend a quantity of casein BRP equivalent to 1.25 g of dried substance in 5 mL of water, add 10 mL of 0.1 N sodium hydroxide, and stir for 1 min (determine the water content of casein BRP prior to the test by heating at 60°C in vacuo for 4 h). Add 60 mL of water and stir with a magnetic stirrer until the solution is practically clear. Adjust to pH 8.0 with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid. Dilute to 100.0 mL with water. Use the solution on the day of preparation.

7. Enterokinase Solution

Dissolve 50 mg of enterokinase BRP in 0.02 M calcium chloride R and dilute to 50.0 mL with the same solvent. Use the solution on the day of preparation.

8. Olive Oil Emulsion

a. Stock emulsion

In an 800-mL beaker 9 cm in diameter, place 40 mL of olive oil R, 330 mL of acacia solution R, and 30 mL of water. Place an electric mixer at the bottom of the beaker. Place the beaker in a vessel containing alcohol R and a sufficient quantity of ice as a cooling mixture. Emulsify using the mixer at an average speed of 1000–2000 r/min. Cool to 5–10°C. Increase the mixing speed to 8000 r/min. Mix for 30 min, keeping the temperature below 25°C by the continuous addition of crushed ice into the cooling mixture (a mixture of calcium chloride and crushed ice is also suitable). Store the stock emulsion in a refrigerator and use within 14 days. The emulsion must not separate into two distinct layers. Check the diameter of the globules of the emulsion under a microscope. At least 90% have a diameter below 3 μm, and none has a diameter greater than 10 μm. Shake the emulsion thoroughly before preparing the emulsion substrate.

b. Olive oil emulsion

For 10 determinations, mix the following solutions in the order indicated: 100 mL of stock emulsion, 80 mL of tris(hydroxymethyl)-aminomethane solution R1, 20 mL of a freshly prepared 8% m/V solution of sodium taurocholate BRP and 95 mL of water. Use on the day of preparation.

9. Starch Solution

To a quantity of starch BRP equivalent to 2.0 g of the dried substance add 10 mL of water and mix (determine the water content of starch BRP prior to the test by heating at 120°C for 4 h). Add this suspension, stirring continuously, to 160 mL of boiling water. Wash the container several times with successive quantities, each of 10 mL, of water and add the washings to the hot starch solution. Heat to boiling, stirring continuously. Cool to room temperature and dilute to 200 mL with water. Use the solution on the day of preparation.

10. Tris(hydroxymethyl)aminomethane [$C_4H_{11}NO_3$ (M_r 121.1); trometamol]

Colorless crystals, very soluble in water, soluble in alcohol, slightly soluble in acetone and in ether. mp: about 170°C.

11. Tris(hydroxymethyl)aminomethane solution R1

Dissolve 60.6 mg of tris(hydroxymethyl)aminomethane R and 0.234 g of sodium chloride R in water and dilute to 100 mL with the same solvent.

Store at 2–8°C and use within 3 days.

12. Maleic anhydride [$C_4H_2O_3$ (M_r 98.1). 2,5-Furandione]

White crystals, soluble in water forming maleic acid, very soluble in acetone and in ethyl acetate, freely soluble in chloroform and in toluene, soluble in alcohol with ester formation, very slightly soluble in light petroleum. mp: about 52°C.

II. CHYMOTRYPSIN (Eur. Pharm.; F.I.P.)

Chymotrypsin is a proteolytic enzyme obtained by the activation of chymotrypsinogen extracted from the pancreas of beef (*Bos taurus* L.). It has an activity not less than 5.0 microkatals per milligram. In solution it has maximal enzymic activity at about pH 8; the activity is reversibly inhibited at pH 3, at which pH it is most stable.

Chymotrypsin is prepared under conditions designed to minimize microbial contamination.

A. Assay

The activity of chymotrypsin is determined by comparing the rate at which it hydrolyzes acetyltyrosine ethyl ester R with the rate at which chymotrypsin BRP hydrolyzes the same substrate under the same conditions.

1. Apparatus

Use a reaction vessel of about 30-mL capacity provided with:

A device that will maintain a temperature of 25.0 ± 0.1°X
A stirring device, for example, a magnetic stirrer
A lid with holes for the insertion of electrodes, the tip of a burette, a tube for the admission of nitrogen, and the introduction of reagents

An automatic or manual titration apparatus may be used. For the latter the burette is graduated in increments of 0.005 mL and the pH meter is provided with a wide scale and glass-calomel electrodes.

2. Test Solution

Dissolve 25.0 mg of the substance to be examined in 0.001 N hydrochloric acid and dilute to 250.0 mL with the same acid.

3. Reference Solution

Dissolve 25.0 mg of chymotrypsin BRP in 0.001 N hydrochloric acid and dilute to 250.0 mL with the same acid.

Store the solutions at 0–5°C. Warm 1 mL of each solution to about 25°C over 15 min and use 50 µL of each solution (corresponding to about 25 nanokatals) for each titration. Carry out the titration in an atmosphere of nitrogen. Transfer 10.0 mL of 0.01 M calcium chloride solution R to the reaction vessel and, while stirring, add 0.35 mL of 0.2 M acetyltyrosine ethyl ester solution R. When the temperature is steady at 25.0 ± 0.1°C (after about 5 min) adjust the pH to exactly 8.0 with 0.02 N sodium hydroxide. Add 50 µL of the test solution (equivalent to about 5 µg of the substance to be examined) and start a timer. Maintain pH at 8.0 by the addition of 0.02 N sodium hydroxide, noting the volume added every 30 s. Calculate the volume of 0.02 N sodium hydroxide used per second between 30 and 210 s. Carry out a titration in the same manner using the reference solution and calculate the volume of 0.02 N sodium hydroxide used per second.

Calculate the activity in microkatals per milligram using the expression:

$$\frac{m' \times V}{m \times V'} \times A$$

where:

m = mass in milligrams of the substance to be examined
m′ = mass in milligrams of chymotrypsin BRP
V = volume of 0.02 N sodium hydroxide used per second by the test solution
V′ = volume of 0.02 N sodium hydroxide used per second by the reference solution
A = activity of trypsin BRP in microkatals per milligram

B. Reagents

1. Acetyltyrosine Ethyl Ester [$C_{13}H_{17}NO_4H_2O$ (M_r 269.3); ethyl *N*-acetyl-L-tyrosinate monohydrate; ethyl (*S*)-2-acetamido-3-(-4-hydroxyphenyl)-propionate monohydrate]

A white, crystalline powder suitable for the assay of chymotrypsin.

α_D^{20}: +21° to +25°, determined on a 1.0% m/V solution in alcohol R.
$A_{1\,cm}^{1\%}$: 60–68, determined at 278 nm using a solution in alcohol R and calculated with reference to the dried substance

2. Acetyltyrosine Ethyl Ester Solution, 0.2 M

Dissolve 0.539 g of acetyltyrosine ethyl ester R in alcohol R and dilute to 10.0 mL with the same solvent.

3. Calcium Chloride Solution

A 7.35% m/V solution.

4. Alcohol

Contains not less than 95.1% V/V and not more than 96.9% V/V of C_2H_6O (M_r 46.07).

A clear, colorless, flammable liquid, mobile, miscible with water, with acetone, with chloroform, with ether, and with glycerol.

d_{20}^{20}: 0.8050–0.8123
bp: 78–79°C.

III. TRYPSIN (Eur. Pharm.; F.I.P.)

Trypsin is a proteolytic enzyme obtained by the activation of trypsinogen extracted from the pancreas of healthy mammals. It has an activity of not less than 0.5 microkatals per milligram, calculated with reference to the dried substance. In solution, it has maximum enzymic activity at pH 8; the activity is reversibly inhibited at pH 3, at which pH it is most stable.

Trypsin is prepared in conditions designed to minimize the degree of microbial contamination.

A. Assay

The activity of trypsin is determined by comparing the rate at which it hydrolyzes benzoylarginine ethyl ester hydrochloride R with the rate at which trypsin BRP hydrolyzes the same substrate in the same conditions.

1. Apparatus

Use a reaction vessel of about 30 mL capacity provided with:

A device that will maintain a temperature of 25.0 ± 0.1°C
A stirring device (e.g., a magnetic stirrer)
A lid with holes for the insertion of electrodes, the tip of a burette, a tube for the admission of nitrogen, and the introduction of reagents.

An automatic or manual titration device may be used. For the latter, the burette is graduated in 0.005 mL and the pH meter is provided with a wide-range scale and glass-calomel electrodes.

2. Test Solution

Dissolve enough of the substance to be examined in 0.001 N hydrochloric acid and dilute to 25.0 mL with the same acid in order to obtain a solution containing approximately 700 nanokatals per milliliter.

3. Reference Solution

Dissolve 25.0 mg of trypsin BRP in 0.001 N hydrochloric acid and dilute to 25.0 mL with the same acid.

Store the solutions at 0–5°C. Warm 1 mL of each solution to about 25°C over 15 min and use 50 μL of each solution for each titration. Carry out the titration in an atmosphere of nitrogen. Transfer 10.0 mL of borate buffer solution pH 8.0 (0.0015 M) R to the reaction vessel and, while stirring, add 1.0 mL, of a freshly prepared 0.686% m/V solution of benzoylarginine ethyl ester hydrochloride R.

When the temperature is steady at 25.0 ± 0.1°C (after about 5 min), adjust the pH to exactly 8.0 with 0.1 N sodium hydroxide. Add 50 µL of the test solution and start a timer. Maintain the pH at 8.0 by the addition of 0.1 N sodium hydroxide the tip of the microburette being immersed in the solution; note the volume added every 30 s. Follow the reaction for 8 min. Calculate the volume of 0.1 N sodium hydroxide used per second. Carry out a titration in the same manner using the reference solution and calculate the volume of 0.1 N sodium hydroxide used per second.

Calculate the activity in microkatals per milligram using the expression:

$$\frac{m' \times V}{m \times V'} \times A$$

where:

m = mass in milligrams of the substance to be examined
m′ = mass in milligrams of trypsin BRP
V = volume of 0.1 N sodium hydroxide used per second by the test solution
V′ = volume of 0.1 N sodium hydroxide used per second by the reference solution
A = activity of trypsin BRP in microkatals per milligram

B. Reagents

1. Benzoylarginine Ethyl Ester Hydrochloride [$C_{15}H_{23}ClN_4O_3$ (M_r 342.8); *N*-benzoyl-L-arginine ethyl ester hydrochloride; ethyl (*S*)-2-benzamido-5-guanidinovalerate hydrochloride]

A white, crystalline powder, very soluble in water and in ethanol, practically insoluble in ether.

$[\alpha]_D^{20}$: −15 to −18°, determined on a 1% m/V solution.
mp: about 129°C.
$A_{1cm}^{1\%}$: 310–340, determined at 227 nm using a 0.001% m/V solution

2. Borate Buffer Solution, pH 8.0 (0.0015 M)

Dissolve 0.572 g borax ($Na_2B_4O_7 \cdot 10\ H_2O$) and 2.94 g calcium chloride ($CaCl_2 \cdot 2H_2O$) in approximately 800 mL of distilled water and titrate with N hydrochloric acid (approximately 2.5 mL) to pH 8.0. Dilute to 1 liter with redistilled water.

IV. PEPSIN POWDER (Eur. Pharm.; F.I.P.)

Pepsin powder is prepared from the gastric mucosa of pigs, cattle, or sheep. It contains gastric proteinases, active in acid medium (pH 1–5). It has an activity not less than 0.5 pH. Eur. U. per milligram, calculated with reference to the dried substance. Pepsin powder is prepared in conditions designed to minimize the degree of microbial contamination.

A. Assay

The activity of pepsin powder is determined by comparing the quantity of peptides nonprecipitable by trichloroacetic acid solution R and assayed using the phosphomolybdotungstic reagent R which are released per minute from a substrate of hemoglobin solution R with the quantity of such peptides released by pepsin powder BRP from the same substrate in the same conditions.

Avoid shaking and foaming during preparation of the test and reference solutions.

1. Test Solution

Immediately before use, prepare a solution of the substance to be examined expected to contain 0.5 Ph. Eur. U. per milliliter in dilute hydrochloric acid R2; before dilution to volume, adjust to pH 1.6 ± 0.1, if necessary, using 1 N hydrochloric acid.

2. Reference Solution

Less than 15 min before use, prepare a solution of pepsin powder BRP containing 0.5 Ph. Eur. U. per milliliter in dilute hydrochloric acid R2; before dilution to volume, adjust to pH 1.6 ± 0.1, if necessary, using 1 N hydrochloric acid.

Place flasks of trichloroacetic acid solution R and hemoglobin solution R in the water bath to equilibrate at 25 ± 0.1°C. Place three tubes in a rack for the reference solutions marked S_1, S_2, S_3, and three blanks marked B_{S1}, B_{S2}, B_{S3} provided with footed stirring rods. Place in another rack three tubes for the test solutions marked T_1, T_2, T_3 and three blanks marked B_{T1}, B_{T2}, B_{T3}. Place the racks in the water bath at 25 ± 0.1°C.

Place in tubes S_1, S_2, S_3 and B_{S1}, B_{S2}, B_{S3} 1.0 mL of the reference solution and in tubes T_1, T_2, T_3 and B_{T1}, B_{T2}, B_{T3} 1.0 mL of the test solution. Equilibrate to 25 ± 0.1°C.

Add 10.0 mL of trichloroacetic acid solution R to tubes B_{S1}, B_{S2}, B_{S3} and B_{T1}, B_{T2}, B_{T3}.

At zero time add 5.0 mL of hemoglobin solution R successively and at 30-s intervals to tubes S_1, S_2, S_3 and T_1, T_2, T_3. Mix the solutions by gently stirring.

Add 5.0 mL of hemoglobin solution R to tubes B_{S1}, B_{S2}, B_{S3} and B_{T1}, B_{T2}, B_{T3} and mix.

Exactly 10 min after adding the hemoglobin solution R and at intervals of 30 s, stop the reaction by adding 10.0 mL of trichloroacetic acid solution R to tubes S_1, S_2, S_3, T_1, T_2, and T_3, and mix.

Filter the contents of each tube (samples and blanks) twice through the same suitable filter paper* previously washed with a 5.0% m/V solution of trichloroacetic acid R, then with water and dried. Discard the first 5 mL of filtrate. Place 3.0 mL of each filtrate separately in a tube containing 20 mL of water. Mix.

Add to each tube 1.0 mL of sodium hydroxide solution R and 1.0 mL of phosphomolybdotungstic reagent R, beginning with the blanks and then the samples of each set, in a known order.

After at least 15 min measure the absorbance of solutions S_1, S_2, S_3 and T_1, T_2, T_3 at 540 nm using the corresponding blank solutions as compensation liquids. The test is not valid unless the absorbances measured are 0.30–0.40. Calculate the mean absorbance for solutions S_1, S_2, S_3 and for solutions T_1, T_2, T_3.

B. Reagents

1. Bromine [Br_2 (M_r 159.8)]

A brownish-red fuming liquid, slightly soluble in water, soluble in alcohol, in chloroform, and in ether.

d_{20}^{20} about 3.1.

2. Hemoglobin

Nitrogen: 15–16%
Iron: 0.2–0.3%
Loss on drying: Not more than 2%
Sulfated ash: Not more than 1.5%

3. Hemoglobin Solution

Transfer 2 g of hemoglobin R to a 250-mL beaker and add 75 mL of dilute hydrochloric acid R2. Stir until solution is complete. Adjust to pH 1.6 ± 0.1 using 1 N hydrochloric acid. Transfer to a 100-mL flask with the aid of dilute

*A suitable filter paper complies with the following test: filter 5 mL of a 5.0% m/V solution of trichloroacetic acid R through a 7-cm disc of white filter paper; the absorbance of the filtrate, measured at 275 nm using unfiltered trichloroacetic acid solution R as the compensation liquid, is less than 0.04.

hydrochloric acid R2. Add 25 mg of thiomersal R. Prepare daily, store at 5 ± 3°C, and readjust to pH 1.6 before use.

4. Hydrochloric Acid, Dilute R2

Dilute 30 mL of 1 N hydrochloric acid to 1000 mL with water; adjust to pH 1.6 ± 0.1.

5. Lithium Sulfate [Li_2SO_4,H_2O (M_r 128.0); dilithium sulfate monohydrate]

Colorless crystals, freely soluble in water, practically insoluble in alcohol.

6. Methyl Orange [$C_{14}H_{14}N_3NaO_3S$ (M_r 327.3); Color index No. 13025; Schultz No. 176; sodium 4'-dimethylaminoazobenzene-4-sulfonate]

An orange-yellow crystalline powder, slightly soluble in water, practically insoluble in alcohol.

7. Methyl Orange Solution

Dissolve 0.1 g of methyl orange R in 80 mL of water and dilute to 100 mL with alcohol R.

Test for sensitivity: A mixture of 0.1 mL of the methyl orange solution and 100 mL of carbon dioxide–free water R is yellow. Not more than 0.1 mL of 0.1 N hydrochloric acid is required to change the color to red.

Color change: pH 3.0 (red) to pH 4.4 (yellow).

8. Phosphomolybdotungstic Reagent

Dissolve 100 g of sodium tungstate R and 25 g of sodium molybdate R in 700 mL of water. Add 100 mL of hydrochloric acid R and 50 mL of phosphoric acid R. Heat the mixture under a reflux condenser in a glass apparatus for 10 h. Add 150 g of lithium sulfate R, 50 mL of water, and a few drops of bromine R. Boil to remove the excess of bromine (15 min), cool, dilute to 1000 mL with water, and filter. The reagent should be yellow in color. If it requires a greenish tint, it is unsatisfactory for use but may be regenerated by boiling with a few drops of bromine R. Care must be taken to remove the excess of bromine by boiling.

Store at 2–8°C.

9. Sodium Hydroxide [NaOH (M_r 40.00)]

Contains not less than 97.0% of total alkali, calculated as NaOH, and not more than 2.0% of Na_2CO_3.

Crystalline white masses, very hygroscopic, readily absorbing carbon dioxide. Supplied in the form of pellets, sticks, or slabs. Very soluble in water, freely soluble in alcohol.

A 1% m/V solution is strongly alkaline and gives the reactions of sodium.

10. Sodium Hydroxide Solution

Dissolve 20.0 g of sodium hydroxide R in water and dilute to 100.0 mL with the same solvent. Verify the concentration by titration with 1 N hydrochloric acid, using methyl orange solution R as indicator, and adjust if necessary to 20.0% m/V.

11. Sodium Molybdate [Na_2MoO_4, $2H_2O$ (M_r 242.0); disodium molybdate dihydrate]

A white, crystalline powder or colorless crystals, freely soluble in water.

12. Sodium Tungstate [$Na_2WO_42H_2O$ (M_r 329.9); disodium tungstate dihydrate]

A white, crystalline powder or colorless crystals, freely soluble in water forming a clear solution, practically insoluble in alcohol.

13. Thiomersal [$C_9H_9HgNaO_2S$ (M_r 404.8); mercurothiolate sodium; ethyl (2-mercaptobenzoato-*S*)mercury sodium salt]

A light, yellowish-white, crystalline powder, freely soluble in water, freely soluble in alcohol, practically insoluble in ether.

14. Trichloroacetic Acid [$C_2HCl_3O_2$ (M_r 163.4)]

Colorless crystals or a crystalline mass, very deliquescent, very soluble in water, in alcohol, and in chloroform.

Store in an airtight container.

15. Trichloroacetic Acid Solution

Dissolve 40.0 g of trichloroacetic acid R in water and dilute to 1000.0 mL with the same solvent. Verify the concentration by titration with 0.1 N sodium hydroxide and adjust if necessary to 4.0 ± 0.1% m/V.

16. Water, Carbon Dioxide–Free

Water that has been boiled for a few minutes and protected from the atmosphere during cooling and storage.

V. HYALURONIDASUM (Eur. Pharm.; F.I.P.)

Hyaluronidase is an enzyme extracted from mammalian testes (e.g., bovine testes) and capable of hydrolyzing mucopolysaccharides of the hyaluronic acid type. It contains not less than 300 I.U. of hyaluronidase activity per milligram, calculated with reference to the dried substance. It may contain a suitable stabilizer.

A. Assay

The activity of hyaluronidase is determined by comparing the rate at which it hydrolyzes sodium hyaluronate BRP with the rate obtained with the International Standard or a reference preparation calibrated in I.U. using a slope-ratio assay.

1. Substrate Solution

To 0.10 g of sodium hyaluronate BRP in a 25-ml conical flask slowly add 20.0 mL of water at 4°C. The rate of addition must be slow enough to allow the substrate particles to swell (about 5 min). Maintain at 4°C and stir for at least 12 hr. Store at 4°C and use within 4 days.

2. Test Solution

Dissolve a suitable amount of the substance to be examined in hyaluronidase diluent R so as to obtain a solution containing 0.6 ± 0.3 I.U. of hyaluronidase per milliliter.

3. Reference Solution

Dissolve a suitable amount of hyaluronidase BRP in hyaluronidase diluent R so as to obtain a solution 0.6 I.U. of hyaluronidase per milliliter.

In a reaction vessel, mix 1.50 mL of phosphate buffer solution pH 6.4 R and 1.0 mL of the substrate solution and equilibrate at 37 ± 0.1°C. At time $t_1 = 0$ (first chronometer) add 0.50 mL of the test solution containing E_t mg of the enzyme to be examined, mix, measure the viscosity of the solution using a suitable viscometer*

*The following viscometer has been found suitable: Ubbelohde microviscometer (DIN 51 562, Part 2), capillary type MII, viscometer constant about 0.1 mm^2/s^2.

maintained at 37 ± 0.1°C, and record the outflow time t_2 using a second chronometer (graduated in 0.1-s intervals) several times for about 20 min (read on the first chronometer). Repeat the procedure using 0.50 mL of the reference solution containing E_r mg of hyaluronidase BRP.

Calculate the viscosity ratio from the expression:

$$\eta_r = \frac{k \times t_2}{0.6915}$$

where:

k = the viscometer constant in mm^2/s^2 indicated on the viscometer
t_2 = the outflow time (in seconds) of the solution
0.6915 = the kinematic viscosity in mm^2/s of the buffer solution at 37°C

Since the enzymatic reaction continues during the outflow time measurements, the real reaction time equals $t_1 + t_2/2$, half of the outflow time ($t_2/2$) for which a certain measurement is valid being added to the time t_1 at which the measurement is started. Plot in $(\ln \eta_r)-1$ as a function of the reaction time $[(t_1 + t_2/2)$ in seconds. A linear relationship is obtained. Calculate the slope for the substance to be examined (b_t) and the reference preparation (b_r).

Calculate the specific activity in I.U. per milligram from the expression:

$$\frac{b_t}{b_r} \times \frac{E_r}{E_t} \times A$$

where:

A = the specific activity of hyaluronidase BRP in I.U. per milligram

Carry out the complete procedure at least three times and calculate the average activity of the substance to be examined.

B. Reagents

1. Phosphate Buffer Solution, pH 6.4

Dissolve 2.5 g of disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$), 2.5 g of sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$), and 8.2 g of sodium chloride in 950 mL of water. Adjust the pH of the solution to 6.4 with 1 N sodium hydroxide or 1 N hydrochloric acid, if necessary. Dilute to 1000.0 mL with water.

2. Gelatin Hydrolyzed

Dissolve 50 g of gelatin R in 1000 mL of water. Autoclave in saturated steam at 121°C for 90 min and freeze-dry.

3. Hyaluronidase Diluent

Mix 100 mL of phosphate buffer solution pH 6.4 R with 100 mL of water. Dissolve 0.140 g of hydrolyzed gelatin R in the solution at 37°C. Use the solution within 2 hr.

VI. UROKINASE (Eur. Pharm.)

Urokinase is an enzyme, obtained from human urine, that activates plasminogen. It consists of a mixture of low-molecular-mass (LMM) (M_r 33,000) and high-molecular-mass (HMM) (M_r 54,000) forms, the high-molecular-mass form being predominant. The potency is not less than 70,000 I.U. per milligram of protein. It is prepared in conditions designed to minimize microbial (including viral) contamination: in particular, adequate measures to inactivate viruses are taken, such as heating of the substance in solution at 60°C for 10 hr.

A. Assay

The potency of urokinase is determined by comparing its capacity to activate plasminogen to form plasmin with the same capacity of a reference preparation of urokinase calibrated in I.U.: the formation of plasmin is measured by the determination of the lysis time of a fibrin clot in given conditions.

The International Unit is defined as the activity contained in a stated amount of the International Reference Preparation, which consists of freeze-dried urokinase with lactose. The equivalent in International Units of the International Reference Preparation is stated by the World Health Organisation.

Unless otherwise prescribed, use phosphate buffer solution pH 7.4 R containing 3.0% m/V of bovine albumin R for the preparation of the solutions and dilutions used in the assay.

1. Test Solution

Prepare a solution of the substance to be examined expected to have an activity of 1000 I.U. per milliliter.

2. Reference Solution

Prepare a solution of a reference preparation having an activity of 1000 I.U. per milliliter.

Keep the test solution and the reference solution in iced water and use within 6 hr. Prepare three 1.5-fold dilutions of the reference preparation such that the longest clot-lysis time is less than 20 min and the shortest clot-lysis time is greater

than 3 min. Prepare three similar dilutions of the test solution. Keep the solution in iced water and use within 1 h. Use 24 tubes 8 mm in diameter. Label the tubes T_1, T_2, and T_3 for the dilutions of the test solution and S_1, S_2, and S_3 for the dilutions of the reference solution, allocating four tubes to each dilution. Place the tubes in iced water. Into each tube, introduce 0.2 mL of the appropriate dilution, 0.2 mL of phosphate buffer solution pH 7.4 R containing 3.0% m/V of bovine albumin R, and 0.1 mL of a solution of thrombin R having an activity of not less than 20 I.U. per milliliter.

Place the tubes in a water bath at 37°C and allow to stand for 2 min to attain temperature equilibrium. Using an automatic pipette, introduce into the bottom of the first tube 0.5 mL of a 1.0% m/V solution of bovine euglobulins R, ensuring mixing. At intervals of 5 s, introduce successively into the remaining tubes 0.5 mL of a 1.0% n/V solution of bovine euglobulins R. Using a stopwatch, measure for each tube the time in seconds that elapses between the addition of the euglobulin solutions and the lysis of the clot. Plot the logarithms of the lysis times for the substance to be examined and for the reference preparation against the logarithms of the concentration and calculate the activity of the substance to be examined using the usual statistical methods.

The estimated potency is not less than 90% and not more than 111% of the stated potency. The fiducial limits of error ($p = 0.95$) of the estimated potency are not less than 80% and not more than 125% of the stated potency.

B. Reagents

1. Citrated Rabbit Plasma

Collect blood by intracardiac puncture from a rabbit kept fasting for 12 h using a plastic syringe with a No. 1 needle containing a suitable volume of 3.8% m/V solution of sodium citrate R so that the final volume ratio of citrate solution to blood is 1.9. Separate the plasma by centrifugation at 1500–1800 *g* at 15–20°C for 30 min. Use within 4 hr of collection. Store at 0–6°C.

2. Euglobulins, Bovine

Use fresh bovine blood collected in an anticoagulant solution (e.g., sodium citrate solution). Discard any hemolyzed blood. Centrifuge at 1500–1800 *g* at 15–20°C to obtain a supernatant plasma poor in platelets.

To 1 liter of bovine plasma add 75 g of barium sulfate and shake for 30 min. Centrifuge at not less than 1500 to 1800 *g* at 5–20°C and draw off the clear supernatant liquid. Add 10 mL of a solution of aprotinin R containing 0.2 mg per milliliter and shake to ensure mixing. In a container with a minimum capacity of 30 liters in a chamber at 4°C introduce 25 liters of distilled water at 4°C and add

about 500 g of solid carbon dioxide. Immediately add, while stirring, the supernatant liquid obtained from the plasma. A white precipitate will form. Allow to settle at 4°C for 10–15 hr. Remove the clear supernatant solution by siphoning. Collect the precipitate by centrifuging at 4°C. Suspend the precipitate by dispersing mechanically in 500 mL of distilled water at 4°C, shake for 5 min, and collect the precipitate by centrifuging at 4°C. Disperse the precipitate mechanically in 60 mL of a solution containing 0.9% m/V of sodium chloride and 0.09% m/V sodium citrate and adjust the pH to 7.2–7.4 by adding a 1% m/V solution of sodium hydroxide. Filter through a sintered glass filter; to facilitate the dissolution of the precipitate crush the particles of the precipitate with a suitable instrument. Wash the filter and the instrument with 40 mL of the chloride-citrate solution described above and dilute to 100 mL with the same solution. Freeze-dry the solution. The yields are generally 6–8 g of euglobulins per liter of bovine plasma.

Test for suitability: For this test, prepare the solutions using phosphate buffer solution pH 7, 4 R containing 3.0% m/V of bovine albumin R.

Into a test tube 8 mm in diameter placed in a water bath at 37°C introduce 0.2 mL of a solution of a reference preparation of urokinase containing 100 I.U. per milliliter and 0.1 mL of a solution of thrombin R containing 20 I.U. per milliliter. Add rapidly 0.5 mL of a solution containing 10 mg of bovine euglobulins per milliliter. A firm clot will form in less than 10 s. Note the time that elapses between the addition of the solution of bovine euglobulins and the lysis of the clot. The lysis time will not exceed 15 min.

Store protected from moisture at 4°C and use within 1 year.

3. Barbital Buffer Solution, pH 7.4

Mix 50 mL of a solution in water containing 19.44 g per liter of sodium acetate R and 29.46 per liter of barbital sodium R with 50.5 mL of 0.1 N hydrochloric acid, add 20 mL of an 8.5% m/V solution of sodium chloride R, and dilute to 250 mL with water.

4. 0.02 M Phosphate Buffer Solution, pH 8.0

To 50.0 mL of 0.2 M potassium dihydrogen phosphate R add 46.8 mL of 0.2 N sodium hydroxide. Dilute to 500.0 mL with water.

5. Albumin, Bovine

Bovine serum albumin containing about 96% of protein. A white to light yellowish-brown powder. Not more than 3.0% water content.

6. Aprotinin

A polypeptide obtained from bovine tissues and consisting of a chain of 58 amino acids. It inhibits stoichiometrically the activity of several proteolytic enzymes such as chymotrypsin, kallikrein, plasmin, and trypsin.

7. Potassium Dihydrogen Phosphate [KH_2PO_4 (M_r 136.1)]

Colorless crystals, freely soluble in water, practically insoluble in alcohol.

8. Potassium Dihydrogen Phosphate, 0.2 M

A solution of potassium dihydrogen phosphate R containing the equivalent of 27.22 g of KH_2PO_4 in 1000.0 mL.

9. Thrombin

Dried human thrombin. A preparation of the enzyme that converts human fibrinogen into fibrin. It is obtained from liquid human plasma and may be prepared by precipitation with suitable salts and organic solvents under controlled conditions of pH, ionic strength, and temperature.

A yellowish-white powder, freely soluble in a 0.9% m/V solution of sodium chloride forming a cloudy, pale yellow solution.

Store in a sealed, sterile container under nitrogen, protected from light, at a temperature below 25°C.

VII. BROMELAIN (F.I.P.)

A. Principle

A casein solution is incubated for 10 min at 35°C and pH 7.0. The reaction is stopped by adding a protein precipitation reagent (containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid), and the undigested casein is removed by filtration. The amount of peptides remaining in solution is determined spectrophotometrically at 275 nm.

B. Unit Definition

The activity of bromelain is determined by comparing the rate at which it hydrolyzes casein with the rate at which the bromelain FIP standard hydrolyzes the same substrate under the same conditions.

C. Reagents and Apparatus

Water bath at 35 ± 0.1°C
Test tubes—rinsed, steamed out, and dried
Pipettes of 5 mL
Flasks of 25, 100, and 1000 mL
Filter paper (Whatman No. 2; Schleicher and Schüll, Weissband No. 589[2])
Stopwatch giving 0.2 s
Magnetic stirrer
Spectrophotometer (275 nm)

All the water used is redistilled water. All the chemicals used are of analytical grade.

1. Protein-Precipitating Reagent

Dissolve 9 g trichloroacetic acid p.a., 24.8 g sodium acetate · $3H_2O$, and 19.5 mL acetic acid in 500 mL water. This precipitation reagent is used because a better relation between activity and absorbance values was obtained.

2. Filter Paper Control

Determine the suitability of the filter paper by filtering a 5-mL portion of the precipitation reagent through the paper and measuring the absorbance of the filtrate at 275 nm, using unfiltered portion of the same precipitation reagent as control.

The absorbance should not be more than 0.04.

3. Buffer Solution: Tris(hydroxymethyl)aminomethane-HCL, M = 0.05.

Dissolve 6.06 g tris(hydroxymethyl)aminomethane in 600 mL CO_2-free redistilled water. Adjust the pH with diluted hydrochloric acid to pH 7.15 at 25°C and add water to a final volume of 1 liter. At 35°C the pH will be 7.00.

4. Enzyme-Activation Solution

Dissolve 60.5 mg cysteine, 37.2 mg EDTA.$2H_2O$, and 74.6 mg KCl in tris buffer. Adjust, if necessary, the pH to 7.15 and fill up to 100.00 mL with tris buffer. The solution must be used on the day it is prepared.

5. Enzyme Reference Solution

Dissolve a suitable amount of bromelain FIP standard in enzyme-activation solution to obtain a solution with an activity of apparently 0.1–0.12 F.I.P. units/mL. Immediately before use, this solution is equilibrated to 35°C in the water bath.

6. Enzyme Test Solution

Dissolve a suitable amount of the substance to be examined in enzyme-activation solution to obtain a solution with an activity of apparently 0.1–0.12 F.I.P. units/mL. Immediately before use, this solution is equilibrated to 35°C in the water bath.

7. Substrate–Activation Solution

Dissolve 242 mg cysteine and 93 mg EDTA.$2H_2O$ in tris buffer. Adjust, if necessary, the pH to 7.15 and fill up to 25.00 mL with tris-buffer. The solution must be used on the day it is prepared.

8. Substrate Solution

Dissolve 2.5 g casein (e.g., casein for biochemistry Merck 2244, Merck Darmstadt, F.I.P. controlled) by adding 10 mL of water, mixing, and adding 15 mL 0.1 N NaOH. After thorough mixing with a magnetic stirrer for 30 min at 35°C, so that all the casein is dissolved, add about 50 mL tris buffer pH 7.15 (solution 3). Add 10 mL substrate activation solution (solution 7). Adjust the pH to 7.15 at 25°C. The volume is made up to 100 mL with tris buffer solution 3. The solution must be used on the day it is prepared.

D. Procedure

Transfer into test tubes:

	Assay	Blank
Casein (solution 8)	2.5 mL	2.5 mL
Place in a water bath at 35°C until temperature equilibrium is reached		
Enzyme (solution 5 or 6)	2.5 mL	—
Mix and incubate for exactly 10 min (chronometer) at 35°C		
Precipitation reagent (solution 1)	5.0 mL	5.0 mL
Enzyme (solution 5 or 6)	—	2.5 mL

After standing at 35°C for another 30 min, the reaction mixture is filtered through a filter paper. The absorbance of the filtrate is measured in a spectrophotometer at 275 nm in a 1-cm cuvette against the blank.

E. Calculation

Calculate the activity in F.I.P. U/mg using the expression:

$$\frac{m' \times E}{m \times E'} \times A$$

where:

m = mass in milligrams of the substance to be examined
m′ = mass in milligrams of bromelain F.I.P. standard
E = absorbance of the filtrate of the substance to be examined
E′ = absorbance of the filtrate of the bromelain F.I.P. standard
A = activity of bromelain F.I.P. standard in F.I.P. U/mg

VIII. CELLULASE (F.I.P.)

A. Unit Definition

The activity of endocellulase is determined by comparing the rate at which it hydrolyzes hydroxyethylcellulose (F.I.P. controlled) with the rate obtained with the endocellulase F.I.P. standard using a slope-ratio assay.

B. Reagents

1. Substrate Solution—3 g hydroxyethylcellulose/1000 mL

Weigh a quantity of hydroxyethylcellulose corresponding to 3.00 g anhydrous product.

A moisture determination can be carried out in the following manner: Weigh duplicate samples of 5 g to the nearest 0.001 g into previously dried and weighed moisture cans with covers. Place the samples in an oven maintained at 105°C and heat for 4 hr. Cool in a desiccator and weigh.

Return the sample to the oven for 1 hr, cool and weigh as before. If the second dried weight is not within 0.001 g of the first, repeat for 1-hour periods until two subsequent weighings are in agreement.

Immediately after the moisture content has been determined, portions of the same undried hydroxyethylcellulose should be taken for the viscosity solution preparations. Transfer 500 mL of distilled water (4°C) into a beaker. The

hydroxyethylcellulose is added slowly using a fast stirring device. The rate of addition should be slow enough (about 3 min) to avoid formation of lumps. Distilled water (4°C) is further added until a final volume of 1000 mL is reached and agitation is continued until all the particles are homogeneously dispersed.

The substrate particles are further allowed to swell at 4°C for at least 12 hours. The swollen particles are then completely dissolved. One hour before use the solution is swirled again and is brought to 25.0°C. The solution is stable for 4 days when kept at 4°C.

2. Buffer, pH 4.5

50 mL of 0.2 M Na_2HPO_4 solution is adjusted to pH 4.5, with approx. 50 mL 0.1 M citric acid solution.

3. Enzyme Reference Solution

Dissolve a suitable amount of endocellulase F.I.P. standard in ice cold water so as to obtain a solution of 0.03 F.I.P. units of endocellulase per mL.

4. Enzyme Test Solution

Dissolve a suitable amount of the substance to be examined in ice cold water so as to obtain a solution of 0.03 F.I.P. units of endocellulase per mL.

C. Apparatus

Constant temperature water bath equipped with a mixing device (25 ± 0.1°C)
Two stopwatches divided in 0.1 s
Viscosimeter: Jenaer Glaswerke Schott, ref. No. 24501/20, type capill. No. II (green label); measuring range 10^{-5}–10^{-4} m^2/s or 10–100 centiStokes

D. Procedure

In a reaction vessel 17.5 mL of the substrate solution are mixed with 2.3 mL buffer solution and equilibrated at 25.0°C. At time $t_1 = 0$ (first chronometer), 0.2 mL of cold enzyme test solution (4) containing E_t of the enzyme is added, mixed, and the thermostatized viscosimeter is filled with this reaction mixture. The outflow times t_2 are recorded using the second chronometer several times for about 10 min. Repeat the procedure using 0.2 mL of the enzyme reference solution (3) containing E_r mg of endocellulase F.I.P. standard.

E. Calculation of Potency

Since the densities of water and the substrate solution are almost equal, we can calculate the viscosity ratio η_r from the formula:

$$\eta_r = \frac{kt_2}{0.893}$$

where:

k = viscosimetric constant written on the viscosimeter
t_2 = the outflow time in seconds of the reaction mixture
0.893 cSt = the kinematic viscosity of water at 25°C

Since the enzymic reaction proceeds further during the outflow time measurement, the real reaction time equals $t_1 + t_2/2$. Half of the outflow ($t_2/2$) for which a certain measurement is valid is added to the time t_1 at which the measurement started. Plots are made of $(\ln \eta_r)^{-1}$ as a function of the reaction time ($t_1 + t_2/2$) in seconds. A linear relationship is obtained. Calculate the slope for the substance to be examined (b_t) and the endocellulase F.I.P. standard (b_r).

Calculate the specific activity in F.I.P. units per milligram from the expression:

$$\frac{b_t}{b_r} \times \frac{E_r}{E_t} \times A$$

where:

A = the specific activity of endocellulase F.I.P. standard in F.I.P. units per milligram

Carry out the complete procedure at least three times and calculate the average activity of the substance to be examined.

F. Example

1. Experimental Results

Standard $k = 0.1002$ cSt. s^{-1} $E_r = 0.033278$ mg		Unknown $k = 0.09776$ cSt. s^{-1} $E_t = 0.0030214$ mg	
t_1 (s)	t_2 (s)	t_1 (s)	t_2 (s)
139.2	109.3	63.1	115.7
285.3	102.1	216.2	106.9
428.1	96.0	357.7	99.9
561.9	90.8	489.8	94.1
		618.3	89.1

2. Calculations

From the first pair of data (t_1 = 139.2 s; t_2 = 109.3 s) are calculated:

$$t_1 + t_2/2 = 193.85 \text{ s}$$

$$\eta_r = \frac{0.1002 \times 109.3}{0.893} = 12.26$$

$$\ln \eta_r = 2.3026 \log \eta_r = 2.507$$

$$(\ln \eta_r)^{-1} = 0.39893$$

All values are calculated in the same manner:

Standard		Unknown	
$t_1 + t_2/2$ (s)	$(\ln \eta_r)^{-1}$	$t_1 + t_2/2$ (s)	$(\ln \eta_r)^{-1}$
193.85	0.39893	120.95	0.39387
336.35	0.41008	269.65	0.40653
476.10	0.42071	407.35	0.41804
607.30	0.43080	536.85	0.42876
		662.85	0.43904

With linear regression analysis we find:

$b_r = 0.00007698$
Corr. coeff. = 0.99998

$$\frac{60 \times 0.00007698}{0.033278} = 0.139 \text{ F.I.P. units.mg}^{-1}$$

$b_t = 0.00008335$
Corr. coeff. = 0.99997

$$\frac{60 \times 0.00008335}{0.0030214} = 1.655 \text{ F.I.P. units.mg}^{-1}$$

Specific activity of the unknown corrected to the standard activity (0.147 F.I.P. units.mg^{-1}):

$$\frac{0.147 \times 1.655}{0.139} = 1.75 \text{ F.I.P. units.mg}^{-1}$$

$$\frac{b_r \times E_r}{b_r \times E_t} \times A = 1.75 \text{ F.I.P. U.mg}^{-1}$$

In the described F.I.P. method corrections for the kinetic energy are not needed since the velocity of the fluid is low enough and, moreover, an absolute viscosimeter is used.

IX. FUNGAL α-AMYLASE (F.I.P.)

A. Introduction

The choice of the assay method for α-amylase preparations will depend on the source of amylase, its purity, and the kind of accompanying substances.

For routine work, the starch iodine reaction, paper disc or chromogenic substrates are useful because of their speed and convenience. The decrease in iodine-staining capacity during starch breakdown primarily reflects cleavage of large chains and is rather insensitive to rupture of polymers with fewer than 25 glucose units. This procedure, like the more cumbersome viscosimetric procedure, is only sensitive to initial random amylase attack and is insensitive to multiple attack. The total number of bonds broken down can be determined by assaying the total increase in reducing power.

In these methods, the reducing groups liberated by hydrolysis of the glucosidic bonds are estimated by any of the conventional methods for the determination of the reducing sugars. Of these, the Willstätter hypoiodite method and its modifications have often been used. The advantage of this method is that iodine reacts stoichiometrically with reducing groups irrespective of the size of the saccharide and that the total number of glucosidic bonds hydrolyzed is measured. The oxidation by hypoiodite in alkaline solution was criticized because iodine is consumed in side reactions when impure substrates are used. These difficulties are avoided by using a pure soluble starch preparation and by choosing the appropriate alkalinity for the reacting iodine solution. Alkaline copper reagents, ferricyanide, or reagents of the Hagedorn-Jensen type have also found extensive use. They are appropriate for comparing enzyme activities under different conditions, but most of them have less reducing power than equimolar quantities of maltodextrins of different sizes.

3,5-Dinitrosalicylic acid proved to be an excellent oxidizing agent for measuring very low amylase activities. The colorimetric response results from the conversion of 3,5-dinitrosalicylic acid to 3-amino-5-nitro-salicylic acid. The color produced by this reduction reaction is proportional to the actual number of reducing groups generated.

B. Description of Assay Method

1. Principle

In alkaline solution, aldoses are oxidized by iodine to carboxylic acids. Since the reaction is not rapid enough to permit direct titration, the sugar solution is mixed with an excess of iodine in alkaline medium.

After having allowed the solution to stand for 15 minutes, it is acidified and the unconsumed iodine determined. One molecule of aldose consumes 2 equivalents of iodine:

$$C_6H_{12}O_6 + I_2 + 3OH \rightarrow C_6H_{11}O_7 + 2I + 2H_2O$$

2. Unit Definition

The amylolytic activity of *A. oryzae* α-amylase is determined by comparison of the amount of glycosidic linkages broken in the starch to the amount released by a reference amylase under the same experimental conditions. It is expressed in terms of micromoles of glycosidic linkages broken per minute.

3. Reagents and Apparatus

Water bath at 25 ± 0.2°C
pH meter
25-mL burette (± 0.05 mL)
tubes and flasks

All reagents used are of analytical grade. The water used is distilled or of equivalent quality.

a. 0.2 M acetate buffer solution pH 5.8

Dissolve 12 g of acetic acid and 1 g of sodium chloride in about 800 mL of water. Adjust the pH to 5.8 with a sodium hydroxide solution (about 10 N). Complete to 1000 mL water.

b. Starch substrate solution: Amylum solubile

Stir an amount of starch equivalent to 2.0 g of dried substance with 10 mL of water. Add the solution obtained to 160 mL of boiling water, stirring continuously. Rinse the original starch container with a further 10 mL of water, add the washings to the hot starch solution, and heat to boiling point while stirring. Cool to room temperature and dilute with water to 200 mL. Use on the day of preparation.

c. 0.25 N sodium hydroxide

Dilute 250 mL of 1 N sodium hydroxide solution to 1000 mL with freshly boiled and subsequently cooled water.

d. 20% sulfuric acid in water

Mix one volume of sulfuric acid with four volumes of water.

e. 0.05 N sodium thiosulfate

To 500 mL of 0.1 N sodium thiosulfate solution add 500 mL of freshly boiled and subsequently cooled water. Store in tightly closed jars.

f. 1 N hydrochloric acid

g. 0.1 N iodine solution

h. Enzyme solution (reference and test)

Dissolve enzymatic products with buffer solution pH 5.8 so that the solutions give a titration volume of thiosulfate, which should be between 6 and 10 mL. Use within 30 min.

4. Procedure

To a stoppered tube (22 mm diameter, 200 mm long) pour 25.0 mL of starch substrate and 10.0 mL of 0.2 M acetate buffer solution. Stopper the tube, mix the contents, and place in a water bath at 25°C. When the temperature of the mixture has reached 25°C, add 2.0 mL of the enzyme solution and record the time of addition. Mix thoroughly and replace in the water bath. After exactly 10 min, add 4 mL of 1 N hydrochloric acid to stop the reaction. Transfer the contents of the tube to a 300-mL stoppered flask, rinse the tube with 20 mL of water, and add the washings to the flask. While stirring continuously, add 10.0 mL of 0.1 N iodine and immediately afterwards 25.0 mL of 0.25 N sodium hydroxide. Allow to stand in the dark at 15–25°C for 15 min. Add 4 mL of the 20% sulfuric acid solution and titrate using a microburette with 0.05 N sodium thiosulfate. Repeat the procedure but add the 4 mL of N hydrochloric acid before the addition of the solution of the sample being examined.

5. Calculation

Carry out the titration of the reference solution under the same conditions as for the test solution.

The amylolytic activity of the product in units per mg is calculated from the expression:

$$\frac{b-a}{b_r-a_r}\,\frac{P_r}{P}\,A$$

where:

- a = mL of 0.05 N sodium thiosulfate used for the titration of the test solution
- a_r = mL of 0.05 N sodium thiosulfate used for the titration of the amylase reference solution
- b = mL of 0.05 N sodium thiosulfate used for the titration of the test solution, inactivated by hydrochloric acid (blank assay)
- b_r = mL of 0.05 N sodium thiosulfate used for the titration of the amylase reference solution, inactivated by hydrochloric acid (blank assay)
- P = mass in mg of the test product
- P_r = mass in mg of the amylase reference product
- A = activity of the amylase reference product in U/mg

X. LYSOZYME (F.I.P.)

A. General Remarks

This method was originally proposed by D. Shugar. Extensive analysis of the reaction conditions was carried out and the assay method was modified in order to increase the sensitivity.

The assay principle is straightforward: the clearance of a *Micrococcus luteus* suspension caused by lysozyme is monitored at 450 nm and compared to a lysozyme standard of known activity. Expression of lysozyme activity in absolute terms is dependent on the substrate sensitivity. Moreover the light scattering effect of the suspension is determined by the optical geometry of the spectrophotometer. Therefore, absolute turbidity values can only be obtained by calibration of the apparatus with a standard of known turbidity.

1. Substrate

Commercially available *M. luteus* cultures often show distinct differences in sensitivity due to specific conditions of growth, harvesting, and processing. Appropriate substrate pretreatment for obtaining a reproducible and homogeneous suspension is also of utmost importance.

Acetone- or phenol-treated or irradiated cells usually are less sensitive, presumably due to structural modifications in the proteoglycan component.

The F.I.P. assay requires the use of lyophilized, viable *M. luteus* ATCC 4698 cells. This substrate is available at the Centre for Standards as a F.I.P.-controlled reference product. It should be stored at –20°C and used within a year.

The use of high-speed mixing devices for suspending the bacteria is likely to damage the cell walls. Suspensions of good quality can be obtained by triturating the lyophilized bacteria with a small amount of buffer in an agate mortar, filtration of the diluted suspension over filter paper with coarse pore size, and ultrasonication in cooled water at low frequency (50 kHz).

Suspensions showing autolysis should be discarded.

2. Buffer Solution

The maximal activity V_{max} at pH 7.00 as a function of the calculated ionic strength is optimal between 0.11 and 0.14 M. A 0.06 M phosphate buffer is recommended. The calculated ionic strength of phosphate buffers (taking into account the various ionic species at pH 7.00) is considerably higher than the molar concentration.

It has been suggested that NaCl plays a role as activator. However, this study has been performed within a limited ionic strength range. In the F.I.P. assay, no salt is added to the buffer medium.

3. Enzyme:Substrate Ratio

An excess of substrate is required in order to guarantee a constant reaction rate during at least 3 min. In a system with an insoluble component, a molar ratio of enzyme to substrate cannot be considered. The optical density of the substrate is limited to 1.000 at 450 nm for reasons of sensitivity. Consequently, the enzyme concentration should be kept as low as possible. In the present assays a final enzyme concentration of 0.3 mg/L is used.

B. Assay Procedure

1. Muramidasic Activity

The muramidasic activity of an unknown lysozyme sample is determined by comparing the initial lysis rate of a *M. luteus* suspension expressed in absorbance decrease per minute at 450 nm with the lysis rate of the same substrate suspension caused by the lysozyme HCl F.I.P. standard in the same conditions.

For kinetic reasons, the unknown lysozyme sample should be diluted to give an absorbance decrease per minute of the same order of magnitude as the standard.

2. Reagents

a. Phosphate buffer solution

Mix 37 mL of a 0.06 M KH_2PO_4 solution with 63 mL of 0.06 M NA_2HPO_4 solution. Adjust to pH 7.00 with either solution. Phosphate buffers are very sensitive to bacterial growth. They should be stored at 4°C and renewed regularly.

b. Substrate suspension

Triturate 50 mg F.I.P.-controlled, lyophilized *M. luteus* ATCC 4698 in an agate mortar with a few drops of phosphate buffer solution A until no visible lumps are left. Transfer quantitatively over filter paper with coarse pore size into a 100-mL volumetric flask. Dilute with phosphate buffer to an absorbance value below 0.900 at 450 nm.

Eventually the suspension can be ultrasonicated at low frequency for not longer than 6 min. Keep the suspension under permanent magnetic stirring at a constant temperature of 25°C. The suspension should be stable for a few hours. Discard suspensions exhibiting spontaneous autolysis.

The assay of the standard and unknown lysozyme should be performed with the same substrate suspension. In the assay a suspension diluted with phosphate buffer to an absorbance of 0.800 ± 0.050 at 450 nm is used.

c. Lysozyme hydrochloride reference solution

Prepare 100 mL of an enzyme solution of F.I.P. lysozyme HCl in bidistilled water containing approximately 4000 F.I.P. units/mL. Use siliconized glassware to prevent adsorption losses. Use quartz cuvettes suited for UV measurements and perform a cuvette correction. Keep the stock solution at a constant temperature of 25°C. Lysozyme in aqueous solutions is very stable, however, it is recommended to renew the solution for every assay series.

d. Lysozyme test solution

Weigh an appropriate amount of test substance and dissolve in a known volume of double-distilled water. In the case of sparingly soluble powders, 0.01 M HCl is recommended.

The powder can be suspended and kept under magnetic stirring at moderate speed or triturated. Insoluble particles should be removed by centrifugation since lysozyme adsorbs onto filter paper. For analysis of ointments or hydrophobic substances, the water-immiscible components can be extracted with diethyl ether.

3. Apparatus

Water bath thermostated at 25 ± 0.1°C
High-precision spectrophotometer with thermostated cuvette holder
Stopwatch showing 0.2 s

Cuvettes, glassware, syringes, and pipettes must be treated with a bichromate–sulfuric acid mixture, rinsed with double-distilled water, and dried. For turbidity measurements, the use of cuvettes with black sides is recommended in order to minimize the effects of stray light.

4. Procedure

Introduce 3.00 mL of substrate suspension B with an initial absorbance of 0.800 ± 0.050 at 450 nm in a cuvette of 1 cm path length and wait for 5 min until temperature equilibrium at 25°C is reached. Add 10 µL of lysozyme solution c or the lysozyme unknown d with a syringe at time zero and mix by inverting the covered cuvette. Read the absorbance every 15 s for 3 min or use a recorder for continuously monitoring the absorbance decrease. Use the phosphate buffer solution a as blank. Plot absorbance versus time and calculate the initial absorbance decrease per minute from the initial, linear part of the curve, using linear regression or the slope of the tangent. Perform the assays in triplicate and calculate the mean absorbance decrease per minute. Proceed in the same way with the lysozyme test solution, which should be appropriately diluted to give an absorbance decrease per minute in the same order of magnitude as the standard.

Perform a blank assay by adding 10 µL of double-distilled water to the substrate suspension, preferably with a different syringe. The suspension should remain stable. It is desirable to calibrate the syringes and pipettes at regular intervals.

5. Calculation

The activity of the unknown sample is expressed relative to the standard, which has a well-defined activity, assigned by collaborative assay and based on kinetic analysis. The stability of the standard is guaranteed by the Centre for Standards. Sensitivity of substrate batches and spectrophotometer variation are not allowed for in an absolute type of assay or unit definition.

Example of calculation: A lysozyme standard solution with an absorbance of 0.240 at 280 nm and thus containing 91.1 mg/L is prepared, and 10 µL of this solution used in the assay. The total assay volume is 3.010 mL with a

final enzyme concentration of approximately 0.3 μg/mL. In this assay a mean absorbance decrease per minute of, for example, 0.140 at 450 nm could be obtained. However, it must be stressed again that this value is totally dependent on the substrate sensitivity and the optical geometry of the photometer.

Suppose that with 10 μL of an appropriately diluted test solution an absorbance decrease of 0.120 per minute is obtained in an assay in the same conditions and with the same substrate suspension. If the potency of the standard is assigned to be 38 F.I.P. units/μg, the absorbance decrease per minute corresponds to $38 \times 0.911 = 34.62$ F.I.P. units in a volume of 10 μL. For the unknown, it can hence be calculated that 10 μL of test solution corresponds to $(34.62 \times 0.120)/0.140 = 29.67$ F.I.P. units.

Since the dilution factor of the test solution and the exact weight of the unknown sample are known, the amount of F.I.P. units/mg sample can be calculated.

6. Remarks

The assay can be extended for clinical use by constructing a calibration curve of absorbance decrease per minute versus enzyme concentration with known dilutions of the lysozyme standard up to 100 μg/mL (stock concentration).

Pathological serum and urine samples with a high activity should be diluted. The absorbance decrease per minute caused by the sample is measured, and the activity can be read directly from the curve. It should be noted that this activity is expressed in hen egg white lysozyme (HEWL) equivalents instead of human lysozyme, which is about four times as active. However, the use of the HEWL standard in clinical analysis is common practice as long as the activity of nonpathological samples is expressed in the same way for comparison.

XI. MICROBIAL LIPASES (F.I.P.)

A. Principle

The catalytic activity is measured by determining the amount of free fatty acids formed over a fixed time interval from an olive oil emulsion, at 37°C, pH 7.0, and in the presence of 0.025% (final concentration) of sodium taurocholate.

To make sure that all fatty acids are indeed measured, the final titration is carried through to pH 9.0. A blank titration on the substrate emulsion is carried out to measure the amount of titrable material not due to lipase action.

B. Unit Definition

The microbial lipolytic activity is determined by comparing the rate at which a solution of microbial lipase powder hydrolyzes a substrate of olive oil emulsion with the rate at which a solution of microbial lipase F.I.P. standard hydrolyzes the same substrate under the same conditions. It should be clearly stated that a lipase of microbial origin is involved to prevent confusion with the assay for pancreatic lipase.

C. Apparatus and Reagents

High-speed homogenizer (about 0.25 HP and 8000 rpm)
Water bath, 37 ± 0.1°C
Magnetic stirring device, about 300 rpm
Automatic titration device with a 25 ± 0.02 mL burette and a pH meter giving a reading to 0.01 are suitable (or manual titration burette (25 ± 0.02 mL with fine tip)
Reaction vessel, capacity 100 mL, diameter 45 mm, height 85 mm
Gum arabic acacia
Olive oil, neutralized according to the following procedure: First the amount of fatty acids in the olive oil sample is determined: 20 g olive oil is mixed with 20 mL amyl alcohol, which has been titrated to just pink with N/5 alcoholic KOH (phenolphthalein). If fatty acids are present, the solution becomes colorless, and the mixture is again titrated with N/5 alcoholic KOH to just pink. If V mL are needed, $V \times 282 \times 10^{-3}$ gives the percentage fatty acid. Next the fatty acids in the olive oil are neutralized with sodium carbonate; about 2 L of olive oil can conveniently be treated at a time. A 2.5 molar excess of $Na_2CO_3 \cdot 10H_2O$ is dissolved in 1/10 its weight of water. The olive oil is heated to 45°C on a water bath. The carbonate solution is gradually added to the warm oil under gentle stirring to avoid emulsification. Stirring is continued for about 15 min. The mixture is next cooled to room temperature and the supernatant decanted in a separatory funnel. After 20 hours the oil is again decanted through filter paper or centrifuged. Any remaining water may be eliminated by a second filtration. The acidity must be below 0.15%.
1 N sodium hydroxide
Sodium taurocholate, F.I.P. controlled
Calcium chloride, $CaCl_2 \cdot 2H_2O$, pro analysi
Amylum alcohol, primary, puriss
Potassium hydroxide, beads, pro analysi
Sodium carbonate, $Na_2CO_3 \cdot 10\ H_2O$, pro analysi
Sodium chloride, NaCl, pro analysi

D. Solutions

1. Gum Arabic Solution

Dissolve 110 g of gum arabic and 12.5 g calcium chloride in double distilled water and fill up to 1 L. Shake or stir for 30 min at room temperature to complete solution.

Centrifuge at 5.000 rpm for 20 min or filter through a Büchner funnel using Celite as a filter aid.

Store at about 5°C.

2. Substrate Emulsion

Place 130 mL of neutralized olive oil and 400 mL of gum arabic (solution a) in a mixer bowl. Emulsify the mixture at high speed for 15 min, keeping the temperature of the mixture below 30°C. The quality of the emulsion should be checked microscopically: 90% of the droplets should have a diameter of 2–3 μm or less, and the diameter of the remaining 10% should not exceed 10 μm.

The substrate has to be reemulsified prior to its immediate use; in doing so the emulsion can be stored for 3 days at 4°C.

3. Enzyme Reference Solution

The microbial lipase F.I.P. standard is dissolved in a 1% NaCl solution and diluted to obtain a solution of 2.4–3.6 F.I.P. microbial lipase units per mL.

4. Enzyme Test Solution

The substance to be analyzed is dissolved in a 1% NaCl solution and diluted to obtain a solution of 2.4–3.6 F.I.P. microbial lipase units per mL.

5. Sodium Hydroxide Solution, 0.02 N

This solution is prepared daily by diluting 10 mL of N NaOH to 500 mL with recently boiled, redistilled water. The solution is kept in stoppered flasks under a nitrogen atmosphere.

6. Sodium Taurocholate Solution

Dissolve 0.5 g of sodium taurocholate F.I.P. controlled in 100 mL of water.

E. Procedure

1. Automatic Titration

The reaction vessel is equilibrated to 37 ± 0.1°C. Into it are measured:

24 mL substrate emulsion 2
9 mL double distilled water
2 mL sodium taurocholate solution

The pH electrodes and the tip of the burette are immersed in the solution; the pH is adjusted to 6.7 with 0.02 NaOH and to pH 7.0 with 0.02 N NaOH. The automatic burette is zeroed. Now, exactly 5 mL of the enzyme reference solution 3 are added; simultaneously a stopwatch is started; the pH is maintained at 7.0 by automatic titration. After exactly 10 minutes, the pH is abruptly brought up to 9.0 by "manual" addition of titrant. This must be completed within 30 seconds. The burette volume is read and the volume of 0.02 N NaOH consumed is recorded (N_{r1}). The blank solution is handled in the same way, however, the addition of enzyme is immediately followed by titration to pH 9.0. The volume is read again and recorded (N_{r2}).

The procedure is repeated using the enzyme test solution, where N_{t1} and N_{t2} are the volumes of 0.02 N NaOH consumed, N_{t2} referring to the blank titration.

2. Manual Titration

The procedure is the same, only the pH is kept at 7.0 by manual titration with 0.02 N NaOH from a 25-mL burette, divided in 0.02 mL.

F. Calculation

Calculate the activity in F.I.P. units per milligram from the expression:

$$\frac{(N_{t1} - N_{t2}) \times m_r}{(N_{r1} - N_{r2})_1 \times m_t} \times A$$

where:

$N_{t1} - N_{t2}$ = average volume of 0.02 N sodium hydroxide used per minute during the titration of the test suspension
$N_{r1} - N_{r2}$ = average volume of 0.2 N sodium hydroxide used per minute during the titration of the reference suspension
m_t = mass in milligrams of the substance to be examined
m_r = mass in milligrams of the reference preparation
A = activity of microbial lipase F.I.P. standard in F.I.P. units per milligram

XII. PAPAIN (F.I.P.)

A. Principle

The specific activity of papain is determined using the synthetic peptide substrate *N*-benzoyl-L-arginine ethyl ester. Hydrolysis is carried out at pH 7.0 and 25°C, and the consumption of base, necessary to neutralize the liberated acid, is recorded as a function of time.

B. Unit Definition

The activity of papain is determined by comparing the rate at which it hydrolyzes benzoylarginine ethyl ester hydrochloride R with the rate at which papain F.I.P. standard hydrolyzes the same substrate under the same conditions.

C. Reagents

All reagents used are of analytical grade. The water is redistilled.

1. Substrate Solution: 0.06 M *N*-benzoyl-L-arginine ethyl ester

Dissolve 514 mg BAEE · HCl in water, adjust to pH 7.0, and fill up to 25.00 mL.

2. Activation Solution

Dissolve 157.5 mg cysteine · HCl, 63.2 mg EDTA · $2H_2O$, and 1.17 g sodium chloride in water, adjust to pH 7.0, and fill up to 100.00 mL.

3. Enzyme Reference Solution

Dissolve a suitable amount of papain F.I.P. standard in bidistilled water and adjust to pH 7.0 to obtain a solution containing approximately 3 F.I.P. units/mL. This solution is kept in ice water during the assay.

4. Enzyme Test Solution

Dissolve enough of the substance to be examined in bidistilled water and adjust to pH 7.0 to obtain a solution containing approximately 3 F.I.P. units/mL.

For nonsoluble samples of papain, the weighed sample is triturated in distilled water at pH 7.0 and the suspension is centrifuged for 15 min (3000 rpm). The supernatant is stored at 0°C.

5. 0.020 N Sodium Hydroxide

D. Apparatus

Reaction vessel: The reaction vessel is provided with a conventional stirring device (e.g. magnetic stirrer) and the temperature of the vessel is maintained at 25.0°C (±0.1°C). The vessel should be covered with a lid provided with one hole for each of the electrodes, one for the burette tip, one for the nitrogen inlet, and one for the introduction of the reactants.

pH meter: A pH meter accurate to 0.01 unit is necessary.

Thermostatic water bath

Microburette: The burette has a total capacity of 0.5 mL and can deliver accurately 0.001 mL.

Stopwatch, giving up to 0.2 s

E. Procedure

Introduce into the reaction vessel, thermostated at 25.0 ± 0.1°C, successively 5.00 mL activation solution 2 and 0.10 mL enzyme solution 3 or 4. Exclude the atmospheric CO_2 by flushing with nitrogen.

When temperature equilibrium is reached, add 5.00 mL substrate solution 1 and, while stirring, titrate the liberated acid with 0.02 N sodium hydroxide 5, the tip of the microburette being immersed in the solution. Follow the reaction for 5 minutes, maintaining the pH constantly at 7.0.

F. Calculation

The initial rate of uptake of alkali is calculated from the curve, giving the number of µL of base as a function of time.

The activity is calculated using the expression:

$$\frac{m' \times V}{m \times V'} \times A$$

where:

m = mass in milligrams of the substance to be examined

m′ = mass in milligrams of papain F.I.P. standard

V = volume of 0.01 N sodium hydroxide used per second by the test solution

V′ = volume of 0.01 N sodium hydroxide used per second by the reference solution

A = activity of papain F.I.P. standard in units per milligram

G. Notes

When the initial rate does not increase proportionally to the enzyme activity until an initial rate of 50 μL of 0.02 N sodium hydroxide per min in the reaction mixture is obtained, an extrapolation of the rectilinear part of the curve is made in the same way as for the determination of the initial rates.

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